Joana Sofia Barros da Costa

Development and application of molecular-based methods for the detection of tree nut allergens: the cases of almond, hazelnut and walnut

Porto

2013

U.PORTO
FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

PhD Thesis

Development and application of molecular-based methods for the detection of tree nut allergens: the cases of almond, hazelnut and walnut

Joana Sofia Barros da Costa

Thesis submitted to Faculdade de Farmácia do Universidade do Porto for Doctor Degree in Pharmaceutical Sciences - Nutrition and Food Science Specialty

Supervised by:

Professor Dr. Maria Beatriz Prior Pinto Oliveira

Dr. Isabel Maria Sousa Gomes Mafra

Porto

December, 2013

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011 and to the PhD grant (SFRH/BD/64523/2009) financed by Programa Operacional Potencial Humano - Quadro de Referência Estratégico Nacional - Tipologia 4.1 - Formação Avançada (POPH-QREN) subsidised by Fundo Social Europeu (FSE) and national funds from Ministério da Ciência, Tecnologia e Ensino Superior (MCTES). In addition to the PhD Grant, the candidate also benefit from supplementary funding to perform a traineeship abroad (3 months in Austria) and to participate in international congresses and conferences.

The work presented in this thesis was mostly performed in the Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto. A part of the research was developed in the Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants, Center of Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Austria.

ACKNOWLEDGEMENTS

My special acknowledgements:

To Professor Dr. Beatriz Oliveira, for her scientific knowledge, encouragement and warm welcome that were always a constant, for her interest and support that made possible the completion of this thesis.

To Dr. Isabel Mafra, for sharing her scientific knowledge, constant availability and enthusiasm in making me pursuit a better work, for her friendship and great personal sacrifice, which were indispensable to the completion of this work and thesis.

To Fundação para a Ciência e a Tecnologia (FCT) by the PhD grant (SFRH/BD/64523/2009) financed by Programa Operacional Potencial Humano - Quadro de Referência Estratégico Nacional - Tipologia 4.1 - Formação Avançada (POPH-QREN) subsidised by Fundo Social Europeu (FSE) and national funds from Ministério da Ciência, Tecnologia e Ensino Superior (MCTES), which made possible the accomplishment of the present work.

To all collaborators from the Laboratory of Bromatology and Hydrology I would like to give a special thanks for the warm welcome and full integration in the team, making my working days much more pleasant, in particular to Joana Santos, Telmo Fernandes, Graciete Santos, Bruna Campos and Ivone Almeida, for their company and sympathy, for sharing the same hopes and for understanding the meaning of pursuing an endless goal. To the Professors, for their kind support and sympathy. And to Anabela Costa, Anabela Borges and Eulália Mendes, for their company and sympathy.

To Professor Dr. Sabine Baumgartner, for accepting me in her laboratory (IFA-Tulln), sharing her knowledge about MS platforms and ELISA systems, her sympathy, support and all the help during my traineeship in Tulln. To Professor Dr Rudolph Krska, Head of the Department for Agrobiotechnology (IFA-Tulln), for his warm welcome and making me part of the team. To Dr. Parisa Ansari, for sharing her scientific knowledge regarding MS platforms, for her kind attention and all the help. To all the friends in IFA-Tulln, for their sympathy and fellowship (especially to Katharina Köstlbauer, Antonia Tacconi and Kathrin Lauter).

To my parents and my sister for their love, support, encouragement and endless patience not only during the course of this PhD but in all the days of my life. They are truly my rock and my safe harbour!

To my all my dearest friends, for their patience, friendly shoulder, support and above all their irreplaceable friendship.

To all the people that were not mentioned here but in some way help me in this conquest.

Research is an endless journey… which I hope has just begun!...

ABSTRACT

Food-induced allergies are defined as adverse health reactions arising from a specific immune response that occurs reproducibly on exposure to a given food. In the past years, food allergies have become an increasing public health concern with an estimated prevalence of 1-2% up to 10% of general population, which represents 70-700 million of individuals all over the world. In theory, any food can induce an abnormal immunological response, still about 90% of the allergic reactions are attributed to specific classes of foods (eggs, milk, fish, crustaceans, soybean, cereals containing gluten, peanut and tree nuts). In general, the clinical symptoms triggered by food allergic reactions can vary from mild to potentially life-threatening. Tree nuts are often associated to the most severe clinical presentations such as anaphylaxis, being therefore, required to be labelled in the pre-package foods since it is the only effective means of protecting the sensitised individuals. To help food industry in the management of food allergens and to assist regulatory authorities in the control of allergenic foods, the development of highly sensitive and specific analytical tools is of utmost importance.

The aim of this work was the development of novel, reliable and highly sensitive DNA-based methods to detect tree nuts as food allergens, namely almond, hazelnut and walnut. To accomplish this goal, real-time polymerase chain reaction (PCR) was the main exploited technique to detect and quantify trace amounts of tree nuts in processed foods. Since most methods for food allergen detection are still based on immunoassays and mass spectrometry (MS) has emerged as a powerful alternative, this work intended also to develop protein-based methods for comparative purposes.

During the course of this work, three methods based on the innovative approach of single-tube nested real-time PCR were successfully developed to detect minute amounts of the three nuts under study. The methods proved to be highly sensitive and specific since they enabled relative and absolute limits of detection of 10-50 mg/kg and 1-4 DNA copies, respectively, being at least one level or order of magnitude lower than the values obtained with conventional real-time PCR and effectively applied to processed foods. A different approach based on the new high resolution melting analysis was also successfully advanced to distinguish closely related species of fruits, namely to discriminate almond from peach, apricot and nectarine.

Because chocolates are foods susceptible of containing tree nuts, they are often excessively labelled as a precautionary practise, restricting their consumption by allergic individuals. Additionally, chocolates are considered very complex matrices, which do not facilitate the development of appropriate tools for their analysis. Therefore, special attention was given to the development of adequate tools to detect hazelnut and almond allergens in chocolates. The critical comparison and evaluation of several different DNA extraction protocols showed that Nucleospin food kit, with minor adjustments, revealed to be the most suitable for quantitative real-time PCR amplification applied to almond and hazelnut in model chocolates, while others failed this task. In the case of hazelnut detection in chocolate, the performance of application of real-time PCR, Enzyme-Linked Immunosorbent Assay (ELISA) and Liquid Chromatography (LC) with MS/MS were critically evaluated. ELISA was successfully developed, but sensitivity was affected by the interfering compounds present in chocolate matrix. LC-MS/MS method was advanced to identify hazelnut allergenic peptides (Cor a 8, Cor a 9 and Cor a 11) with high sensitivity in model chocolates. Comparative assessment showed that reliable sensitivities levels were found to be similar among protein- and DNA-based methods.

In summary, with the presented work, new contributions were proposed using different DNA- and protein-based approaches for the better management of allergenic food ingredients, some of them with potential application to food industry. In addition, the proposed new tools and achieved results will contribute for a prospective harmonisation of methods for tree nut allergen analysis and for the control of food allergens by the regulatory authorities.

Keywords: food allergies, real-time PCR, ELISA, novel molecular techniques.

RESUMO

As alergias alimentares definem-se como reacções adversas decorrentes de uma resposta imunológica específica, que ocorre de forma reprodutível em indivíduos sensibilizados, como resultado da exposição a um dado alimento. Nos últimos anos, as alergias alimentares tornaram-se numa preocupação crescente de saúde pública, com uma prevalência estimada de 1-2% a 10% da população geral, o que representa que cerca de 70-700 milhões de pessoas em todo o mundo possam sofrer de algum tipo de alergia alimentar. Em teoria, qualquer alimento pode induzir uma resposta imunológica adversa, no entanto estima-se que cerca de 90% das reações alérgicas possam ser causadas por alimentos que estão reunidos em oito grupos de alimentos específicos (ovos, leite, peixes, crustáceos, soja, cereais contendo glúten, amendoim e frutos de casca rija). Em geral, as reacções alérgicas alimentares induzem o aparecimento de sintomas clínicos que podem variar de ligeiros a potencialmente fatais. De entre os alimentos classificados como virtualmente alergénicos, os frutos de casca rija são frequentemente associados a sintomas clínicos graves, tais como a anafilaxia, havendo portanto, a obrigatoriedade de serem sempre rotulados independentemente da sua quantidade, pois é o único meio eficaz de proteger os indivíduos sensibilizados. Neste sentido e com forma a ajudar as indústrias na gestão dos alergénios alimentares e apoiar as entidades reguladoras no controle dos mesmos, o desenvolvimento de ferramentas analíticas com elevada sensibilidade e especificidade, é de extrema necessidade.

O objetivo deste trabalho consistiu no desenvolvimento de novos métodos baseados no ADN, com elevada sensibilidade e especificidade, para a deteção de frutos de casca rija como alergénios alimentares, mais concretamente, para a identificação de amêndoa, avelã e noz. Para tal, a reação em cadeia da polimerase (PCR) em tempo real, foi a principal técnica explorada para detectar e quantificar vestígios de frutos de casca rija em alimentos processados. Uma vez que a maioria dos métodos de deteção de alergénios alimentares ainda se baseiam em ensaios imunológicos e mais recentemente nos métodos de espectrometria de massa (MS) como ferramentas alternativas, este trabalho destinou-se também a desenvolver técnicas à base da deteçao de proteínas para fins comparativos.

No decurso deste trabalho, três métodos baseados numa abordagem inovadora que reúne as vantagens de duas tecnologias diferentes (nested PCR e PCR em tempo real) num único tubo, foram desenvolvidas com êxito para detectar quantidades mínimas dos frutos de casca rija em estudo (amêndoa, avela, noz).

Nos três casos, os métodos provaram ser altamente sensíveis e específicos, uma vez que permitiram atingir limites de detecção relativos e absolutos de 10-50 mg/kg e 1-4 cópias de ADN, respectivamente, sendo pelo menos, um nível ou ordem de magnitude mais baixo do que os valores obtidos com a PCR em tempo real convencional e efectivamente aplicado a alimentos processados. Uma nova e diferente abordagem com base na análise por *high resolution melting* também foi avançada para a distinção efectiva de espécies de frutos geneticamente relacionados, ou seja, para permitir discriminar amêndoa de frutos do género *Prunus* tais como o pêssego, o damasco e a nectarina.

Porque os chocolates são alimentos suscetíveis de conterem frutos de casca rija, são na maioria das vezes excessivamente rotulados como medida de precaução, restringindo assim o seu consumo aos indivíduos alérgicos. Adicionalmente, os chocolates são considerados matrizes alimentares muito complexas, o que dificulta o desenvolvimento de métodos adequados para sua análise. Considerando esta questão, foi dada uma relevância especial ao desenvolvimento de ferramentas adequadas para a deteção de avelã e amêndoa em chocolates. A avaliação crítica e comparação de vários protocolos para a extracção de ADN, mostrou que o kit comercial *NucleoSpin Food*, revelou ser o mais adequado para a amplificação de amêndoa e avelã de chocolates modelo por métodos de PCR qualitativa e PCR em tempo real. No caso da detecção de avelã de chocolates modelo, diferentes metodologias (PCR em tempo real, ensaio imunológicos e cromatografia líquida acoplada a deteção por espectrometria de massa – LC-MS/MS os desempenhos da) foram desenvolvidas, aplicadas e criticamente avaliadas. O método imunológico baseado em enzyme-linked immunosorbent assay (ELISA) foi desenvolvido com sucesso, no entanto a sensibilidade foi afetada pela presença de compostos interferentes na matriz de chocolate. O método de LC-MS/MS com elevada sensibilidade e especificidade foi também avançado para a identificação inequívoca de avelã em chocolates modelo, com alvo nos péptidos alergénicos, Cor a 8, Cor a 9 e Cor a 11. Os níveis de sensibilidade obtidos com as diferentes técnicas (baseadas no ADN e proteinas), foram semelhantes para a deteção de avelã em chocolates modelo.

Em resumo, com o trabalho apresentado, novas metodologias foram avançadas com sucesso, usando diferentes abordagens com base na deteção de ADN e de proteínas, visando uma melhor gestão dos ingredientes alergénicos, tendo alguns deles potencial aplicação à indústria alimentar. Além disso, as novas ferramentas propostas e os resultados obtidos irão contribuir para uma maior harmonização dos métodos de deteção dos alergénios de frutos de casca rija e para um melhor controlo dos alergénios alimentares por parte das autoridades reguladoras.

Palavras-Chave: alergias alimentares, PCR em tempo real, ELISA, novas técnicas moleculares.

LIST OF PUBLICATIONS AND COMMUNICATIONS

(Within the scope of this PhD)

Publications in international peer-review Journals

1. Almond allergens: molecular characterization, detection, and clinical relevance.
   Joana Costa, Isabel Mafra, Isabel Carrapatoso, M. Beatriz P. P. Oliveira
   Journal of Agricultural and Food Chemistry, **2012**, 60, 1337-1349.

2. Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut.
   Joana Costa, Isabel Mafra, Tomas Kuchta, M. Beatriz P. P. Oliveira,
   Journal of Agricultural and Food Chemistry, **2012**, 60, 8102-8110.

3. High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods.
   Joana Costa, Isabel Mafra, M. Beatriz P. P. Oliveira
   Food Chemistry, **2012**, 133, 1062-1069.

4. Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods.
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   Food Research International, **2013**, 51, 228-235.

5. Applicability of a real-time PCR system to verify labelling compliance of nut allergens in chocolates.
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   Clinical and Translational Allergy, **2013**, 3 (Suppl. 3), P129

6. Development of a novel system based on single-tube nested real-time PCR system for the quantification of hazelnut in complex foods.
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   Clinical and Translational Allergy, **2013**, 3 (Suppl. 3), P144

7. Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes.
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   Food Research International, in press (DOI: 10.1016/j.foodres.2013.09.047)

8. Hazelnut allergens: molecular characterization, detection, and clinical relevance.
   Joana Costa, Isabel Mafra, Isabel Carrapatoso, M. Beatriz P. P. Oliveira
   Critical Reviews in Food Science and Nutrition (accepted)

9. Walnut allergens: molecular characterization, detection, and clinical relevance.
   Joana Costa, Isabel Carrapatoso, M. Beatriz P. P. Oliveira, Isabel Mafra
   Clinical and Experimental Allergy (submitted)

10. Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols.
    Joana Costa, Vítor S. Melo, Cristina G. Santos, M. Beatriz P. P. Oliveira, Isabel Mafra
    Food Chemistry (submitted)

11. Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates
    Joana Costa, Parisa Ansari, Isabel Mafra, M. Beatriz P. P. Oliveira, Sabine Baumgartner
    Food Chemistry (submitted)

12. Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR
    Joana Costa, Parisa Ansari, Isabel Mafra, M. Beatriz P. P. Oliveira, Sabine Baumgartner
    Analytical and Bioanalytical Chemistry (submitted)

Publications in national peer-review Journals

1. Alergénios alimentares: O que são, o que provocam e como detetá-los?
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   Química, **2012**, 127, 33-38.

2. Alergénios em produtos da pesca e derivados**.**
   Telmo J. R. Fernandes, Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   Riscos e Alimentos, **2012**, 4 (12), 27-29.

Awards

Part of the work performed during the course of this PhD, regarding the development of novel molecular-based techniques for the assessment of allergens in foods, was presented with an award and a nomination.

- 2nd Award in the FOOD I&DT 2013 promoted by Rede Inovar with project "AlergenControl – Novas abordagens para o controlo de alergénios alimentares".

- Nomination for Nutrition Awards 2013 promoted by the Associação Portuguesa dos Nutricionistas and the GCI (with Governo de Portugal as the institutional partner) with project "AllergenSafeFood – Desenvolvimento de novas ferramentas para a avaliação da segurança de alimentos contendo potenciais alergénios" in the category of Research and Development.

Publications in Proceedings of Scientific Meetings

1. A novel high sensitive approach based on single-tube nested real-time PCR to detect hazelnut allergens.
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   11º Encontro de Química dos Alimentos, 16-19 September 2012, Bragança, Portugal.
   CD-ROM of Proceedings, 1-4.

2. Evaluation of DNA extraction methods to detect almond allergens in chocolates
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   11º Encontro de Química dos Alimentos, 16-19 September 2012, Bragança, Portugal.
   CD-ROM of Proceedings, 1-4.

3. Applicability of single-tube nested real-time PCR as a new technique to detect almond at trace amounts
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra
   11º Encontro de Química dos Alimentos, 16-19 September 2012, Bragança, Portugal.
   CD-ROM of Proceedings, 1-4.

Oral communications in Scientific Meetings

1. Análise por "High Resolution Melting" como uma nova abordagem para a detecção de alergénios da amêndoa
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
   XXII Encontro Nacional SPQ, Braga, Portugal.
   3-6 June 2011.

2. Real-time PCR technique to detect hazelnut in chocolate as a potential hidden allergen
   Vítor S. Melo, Cristina G. Santos, Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
   5th Meeting of Young Researchers of University of Porto (IJUP2012), Porto, Portugal.
   22-24 February 2012.

3. A novel high sensitive approach based on single-tube nested real-time PCR to detect hazelnut allergens
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
   11º Encontro de Química dos Alimentos, Bragança, Portugal.
   16-19 September 2012.

4. Detection of walnut allergens in raw and baked products by molecular based approaches
   Liliane Pinheiro, Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
   6th Meeting of Young Researchers of University of Porto (IJUP2013), Porto, Portugal.
   13-15 February 2013.

5. Novel approaches based on single-tube nested real-time PCR technology for the detection of nut allergens in processed foods
   Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
   4th MoniQA International Conference, Budapest, Hungary.
   26 February-1 March 2013.

6. Effect of baking process on the detection of walnut allergens in cakes by DNA-based approaches
   Joana Costa, Liliane Pinheiro, M. Beatriz P. P. Oliveira, Isabel Mafra.
   8th International Conference on Culinary Arts and Sciences, Porto, Portugal.
   19-21 June 2013.

ACRONYMS

aa – Amino acid

AL60SRP – *Prunus dulcis* 60s acidic ribosomal protein gene

AMP – Almond major allergen

Ana o – Allergens from *Anacardium occidentale* (cashew nut)

Ara h – Allergens from *Arachis hypogaea* (peanut)

Bet v – Allergens from *Betula verrucosa* (birch pollen)

bp – Base pair

Cor a – Allergens from *Corylus avellana* (hazelnut)

CTAB – Cetyltrimethylammonium bromide

CRM – Certified Reference Materials

Ct – Cycle threshold

DBPCFC – Double-Blind Placebo-Controlled Food Challenge

DNA – Deoxyribonucleic acid

EAACI – European Academy of Allergology and Clinical Immunology

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-Linked Immunosorbent Assay

ESI – Electrospray ionization

EU – European Union

FAO – Food and Agriculture Organisation of the United Nations

FAOSTAT – Food and Agriculture Organisation of the United Nations, Statistics Division

GMO – Genetically Modified Organisms

HRM – High Resolution Melting

Hsp – Heat shock proteins

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgY – Immunoglobulin Y

IRMM – Institute for Reference Materials and Measurements

IUIS – International Union of Immunological Societies

Jug r – Allergens from *Juglans regia* (walnut)

Jug n – Allergens from *Juglans nigra* (walnut)

LC-MS – Liquid Chromatography Mass Spectrometry

LFD – Lateral Flow Device

LIT – Linear ion trap

LOAEL – lowest observed adverse effect level

LOD – Limit of Detection

LOQ – Limit of Quantification

LPA – ligation dependent probe amplification

LTP – Lipid Transfer Protein

Mal d – Allergens from *Malus domestica* (apple)

MALDI-TOF – Matrix assisted laser desorption ionisation – time of flight

MIQE – Minimum Information for publication of Quantitative real-time PCR Experiments

NCBI – National Center for Biotechnology Information

NOAEL – no observed adverse effect level

nsLTP – non-specific Lipid Transfer Protein

OAS – Oral Allergy Syndrome

OFC – Oral Food Challenge

OIT – Oral Immunotherapy

PCR – Polymerase Chain Reaction

PNA – Peptide nucleic acid

PR – Pathogen-Related

Pru ar – Allergens from *Prunus armeniaca* (apricot)

Pru av – Allergens from *Prunus avium* (cherry)

Pru du – Allergens from *Prunus dulcis* (almond)

Pru p – Allergens from *Prunus persica* (peach)

PVP – Polyvinylpyrrolidone

Pyr c – Allergens from *Pyrus communis* (pear)

rcbl – Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

RNA – Ribonucleic acid

SDS-PAGE – sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Ses i – Allergens from *Sesamum indicum* (sesame)

SPR – Surface Plasmon Resonance

SPT – Skin Prick Test

SRM – Selected Reaction Monitoring

60sRP – *Prunus dulcis* 60s acidic ribosomal protein P2

Ta – Temperature of annealing

TLP – Thaumatin-Like Protein

Tm – Temperature of melting

USA – United States of America

USDA – United States Department of Agriculture

UV – Ultraviolet radiation

INDEX

GENERAL INTRODUCTION

What is food allergy?

Food-induced allergies are defined as adverse health reactions, arising from specific immune responses that occur reproducibly on exposure to a given food [1]. Normally, this type of reactions affects a small, but rather important portion of the general population that are described as sensitised or allergic individuals, being considered an emergent problem of public health with special emphasis on Western Societies [2]. The severity of an allergic reaction is highly dependent on several factors with a wide variety of clinical presentations among individuals. While for some the ingestion of an offending food represent the occurrence of discomfort symptoms such as itching in the mouth or skin irritations, for others the consumption of the allergenic food can lead them to the nearest emergency with clear signs of anaphylaxis (severe and potentially life-threatening allergic reaction) [1,3].

Food allergies are mediated by the immune system by activating the immunoglobulin E (IgE), although they can also be triggered by non-IgE (e.g. celiac disease), mixed IgE and non-IgE (e.g. eosinophilic gastroenteritis) and cell mechanisms (e.g. allergic contact dermatitis) [1]. From these four categories, IgE-mediated reactions are by far, the most commonly related to abnormal immunological responses induced by allergenic foods.

What are food allergens?

From a physiological point of view, allergens are classified as antigens that have the capacity to initiate an adverse immunological response in sensitised/allergic individuals. Biochemically, food allergens are proteins (mainly glycoproteins) that are water soluble and highly resistant to digestion. In general, the allergenic proteins present low molecular weights (<70 kDa) with acidic isoelectric points that are greatly abundant in the food source. They are usually resistant to proteases, heat and denaturation, allowing maintaining their integrity during food preparation and digestion [4]. Most of the allergens have enzymatic activity, which enable them to cross the mucosal membranes. Subsequently, these proteins are recognised by allergen-specific immune cells (usually the IgE) and elicit precise immunologic reactions that result in characteristic symptoms [1].

Depending on the route of sensitisation, food allergens can be classified as class I or class II [5]. The class I food allergens includes all the allergenic proteins that induce genuine reactivity through the gastrointestinal tract, indicating a direct route of sensitisation via ingestion of the offending proteins. When secondary sensitisation to cross-reactive food allergens occurs as a consequence of the initial reactivity to homologous pollen-related allergens, these proteins are classified as class II [6].

According to this classification, most of class I food allergens are heat stable and resistant to degradation or proteolytic digestion, thus susceptible of inducing severe and systemic reactions. Class II food allergens are usually easily degradable, being mostly likely responsible for triggering mild allergic reactions often limited to symptoms in the oral cavity [7, 8].

Another relevant characteristic of food allergens regards the existence of epitopes that consist of particular groups of amino acids, either in a sequential (linear) or conformational structure, with the ability to bind IgE antibodies [5]. When compared with conformational epitopes, the linear ones seem to be more important in food allergens since food proteins are usually cooked, leading to heat denaturation and alteration in tertiary structure. In addition to food processing, proteins are also digested along the gastrointestinal tract, prompting further alteration and rupture of their conformation before reacting with the immune system. As consequence, it is thought that linear epitopes become more accessible for potential interactions with IgE antibodies, conducting to more severe allergic responses [5]. Linear epitopes have been suggested to be more important in class I food allergy, while conformational epitopes seem to be more relevant in class II food allergy. The conformational epitopes are extremely dependent on the tri-dimensional structure of the allergen, which indicates that they can be simply affected by potential alterations on the native configuration of the proteins. Therefore, conformational epitopes are suggested to be involved in food allergy induced by the consumption of fresh fruits and vegetables. In these cases, inhalant and food allergens present high homology, leading to cross-reactivity phenomena [5].

The extensive study on food allergens has led to the characterisation of their biological functions and further inclusion in different protein families (Table 1). Regarding the animal-derived allergens, the majority is classified as transport (caseins, albumins and globulins) or regulation/structural proteins (tropomyosins and parvalbumins). With respect to plant-derived allergens, they mostly storage proteins (vicilins, albumins and legumins), although some allergens are known to perform different biological functions such as transport (Lipid Transfer Proteins - LTP), structural (profilins) and defence (Pathogen-Related protein - PR-10) [9].

How does the immune system respond to food allergy?

The immune system is usually composed of a complex network of cells and antibodies that are responsible for playing different specific roles in the organism. Therefore, the immune system is prepared to identify harmless environmental substances, inducing immune tolerance to them and protecting the organism from the dangerous ones [3].

Table 1. Representation of the eight groups of foods susceptible of inducing allergic reactions in sensitised individuals, according to their protein families and biochemical function. Examples of identified and characterised animal and plant allergens are provided.

Foods	Protein families	Biochemical function	Examples of allergens
Eggs	Albumins	Transport	Gal d 2
Fish	Parvalbumins	Regulation	Gad c 1 (belong to codfish, but is common to most fish species)
Milk	Globulins Caseins Albumins	Transport Transport Transport/ Regulation	Bos d 8 Bos d 4 Bos d 5
Crustaceans	Tropomyosins	Regulation/Structural	Pen i 1 (shrimp) Cha f 1 (common crab)
Soybean	Legumins Vicilins Profilins PR-10	Storage Storage Structural Defence	Gly m 6 Gly m 5 Gly m 3 Gly m 4
Tree nuts (almond, walnut, cashew, hazelnut, pistachio, pecan nut, macadamia nut and Brazil nut)	PR-10 LTP Profilins Vicilins Legumins 2S albumins	Defence Transport Structural Storage Storage Storage	Cor a 1 (hazelnut), Pru du 1 (almond) Pru du 3 (almond), Cor a 8 (hazelnut) Pru du 4 (almond), Jug r 5 (walnut) Cor a 11 (hazelnut), Jug r 2 (walnut) Car i 4 (pecan nut), Ana o 2 (cashew) Pis v 1 (pistachio), Ber e 1 (Brazil nut)
Peanut	Vicilins 2S albumins Legumins Profilins Oleosins PR-10 LTP	Storage Storage Storage Structural Structural Defence Transport	Ara h 1 Ara h 6/Ara h 7 Ara h 3 Ara h 5 Ara h 10/Ara h 11 Ara h 8 Ara h 9
Wheat	Profilins LTP Thioredoxins Gliadins Glutenins	Structural Transport Transport Storage Storage	Tri a 12 Tri a 14 Tri a 25 Tri a 19/Tri a 21 Tri a 26

(Adapted from Costa et al [9])

Although the reason is not yet fully understood, in some individuals the immunological system produces irregular responses, resulting in the overproduction of IgE antibodies in relation to some food proteins, which cause the appearance of food allergies. The development of an allergy occurs in two stages: sensitisation and reaction. The sensitisation occurs upon the first contact of an individual with a given food and corresponds to the first stage of food allergy. The immune system initiates the production of large amounts of antibodies (IgE) that will specifically recognise the offending food after contact with it. Once the individual has been sensitised, the subsequent exposure to the allergenic food can lead to an allergic reaction, corresponding to the next stage of food allergy. The allergic reaction starts with the activation of mast cells, which will induce the release of several compounds such as histamine, prostaglandins and cytokines, among others (Fig. 1). This process is responsible for triggering the manifestation of clinical symptoms that can vary in intensity, according to the degree of exposure [10].

Sensitisation to a given food, followed by an episode of observable symptoms indicates the development of a food allergy [1].

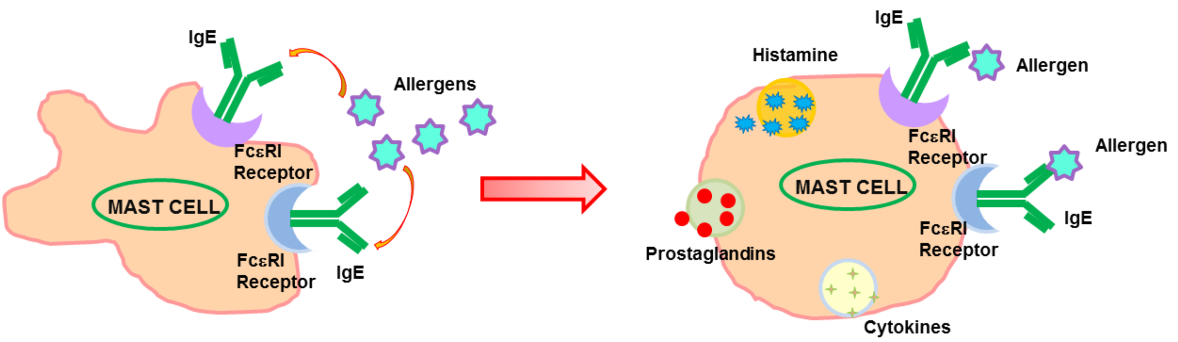


Fig. 1 Schematically representation of the processes activated by the presence of an allergenic food. IgE antibodies bind to the target proteins (allergens) leading to the degranulation of the mast cells and subsequent release of histamine, cytokines, prostaglandins and/or other mediators.

Is the number of food allergic individuals increasing?

Recent studies seem to indicate that food allergies can affect 1-2% up to 10% of general population, estimating an incidence of 3-4% among adults and 5-6% in children/adolescents [11, 12]. In Europe alone, the number of allergic patients is predicted to reach 17 million, which corresponds to almost 3.5% of the European population [3, 13]. Food-induced allergies are estimated to be increasing, especially in the industrialised countries. However, as they are often confused with food intolerances, this rising perception could be overestimated. Although special attention has been dedicated to several issues related to food allergies, topics such as actual prevalence, basis and cost of food allergies are yet to be determined [14].

Virtually any food can be responsible for triggering an allergic reaction, still about 90% of the abnormal immunological responses are attributed to particular classes of foods that are commonly known as the big-8 (eggs, milk, fish, crustaceans, soybean, cereals containing gluten, peanut and tree nuts). Though any of the described groups can induce mild to severe adverse immunological responses, peanuts and tree nuts are often causative of systemic allergic reactions (even potentially fatal). In this context, the allergies induced by tree nuts and peanuts consumption are the main cause for anaphylaxis, leading to high numbers of individuals to emergencies, every year and all around the world [15]. Anaphylaxis can be triggered by any type of food and in individuals of all ages. However, most of the fatalities provoked by anaphylactic shocks occur among adolescents and young adults, owing to the ingestion of allergenic foods such as peanuts and tree nuts. Additionally, in patients with clear clinical diagnosis of respiratory and food-induced allergies, the risk of suffering episodes involving several target systems (anaphylaxis) upon ingestion of the offending food is very high. In those cases, if the individual came across with an accidental exposure to the allergenic ingredient, a small delay in using the epinephrine injection often results in fatality [15].

The specific amount of allergen known to be responsible for an allergic reaction depends on several factors, such as the immunological response of each individual, the time of the day, the pre-existing infections, the practice of exercise and/or stress conditions [16]. The sensitised/allergic patients can also present different degree of tolerance to a given allergen, as a result the minimal doses susceptible of inducing an allergic reaction are yet to be defined. Still, some clinical trials seem to suggest that a quantity as small as 1 mg (or less) of allergenic protein can be sufficient to elicit an abnormal immunological response [17, 18].

So far, the population-based studies regarding the prevalence of food allergies are at a preliminary stage, which highlight a lack of information about this subject. Although food allergy has been classified as a major problem of public health, no effective treatments and certainly no cure, have yet been made available [1, 3]. As consequence, the only actual means of protecting the allergic patients from adverse immunological reactions is the total avoidance of the offending food. Additionally, these individuals are also advised to eliminate from diet any foods that could cause cross-reactivity with the allergenic ingredient, since the frequency of the allergic reactions as result of cross-reactivity is very high. For example, if a patient is allergic to almond, the individual is advised, not only to exclude almond from diet, but also to avoid other nuts (e.g. hazelnut, cashew) or foods from the same botanical family such as peach, apple or apricot [19].

The development of natural tolerance to certain foods can happen during growth. This is the case of allergies to milk or eggs, which are known to present a high incidence among children of young age, though most of them will outgrow their allergies during childhood [20]. In opposition, children presenting allergies induced by other foods such as peanuts and tree nuts are more likely to be persistently affected by them throughout adulthood [20]. Besides all the recommendations and all the precautions implemented to protect these patients, the total elimination of the offending food from diet is almost impossible to maintain. For instance, the annual incidence rate of accidental exposure of children to peanut allergy is 12.5%, being those with a recent diagnosis and adolescents at higher risk [21].

Until now, only some preventive and corrective measures are available to manage food allergy. Thus, when an accidental exposure occurs, some procedures can be undertaken to minimise the damage provoked by food allergies. For minor allergic reactions, the prescription of antihistaminics and corticosteroids is normally recommended since it can help reducing the symptoms [3, 22]. However, these pharmaceuticals are not appropriated to treat a severe allergic reaction. For more severe and systemic reactions (anaphylaxis), the use bronchodilators and/or epinephrine are usually the first-line of treatment. In this context, some research has been conducted aiming at developing better forms to treat food allergies. Oral tolerance induction protocols for some foods (peanut, milk and eggs) are currently under development, still further research is needed to ensure the effectiveness and safety of these treatments [20, 23].

Management of food allergy

Food allergies represent a very relevant issue of food safety, owing to the predictable severe and potential fatal outcomes. As consequence, it was necessary to establish legal basis seeking to protect the sensitised/allergic individuals. Presently, through a set of directives and regulations, the European Union (EU) ruled the mandatory labelling of fourteen groups of certain substances or products causing allergies or intolerances that are required to be emphasised from the rest of the ingredients enumerated in processed foods, regardless of their quantity [24, 25]. In this list of fourteen groups of potentially allergenic foods are included: soybean, gluten-containing cereals, sesame, mustard, celery, peanuts, tree nuts, milk, eggs, fish, molluscs, crustaceans, lupine and sulphites [24, 25]. Although the legal basis for food allergies imposed by the EU is the most complete in respect to the list of 14 groups, other countries such as Canada (11 groups), Australia and New Zealand (10 groups), USA (8 groups) and Japan (6 groups) also establish restrictive legislation to protect the allergic individuals.

Actually, as a result of the growing complexity of food formulations and food processing, foods can be unintentionally contaminated with allergen-containing ingredients or via cross-contamination along the production lines [26]. In this context, not only the allergic patients can be drastically affected, but also food producers and competent authorities involved in controlling food labelling. To address these issues, food industry and regulatory authorities rely on the available analytical methods to determine the amount of a particular allergic ingredient in a food in order to decide about the safety of the product [26].

Considering both legal and ethical perspectives, food industry has to inform food-allergic individuals about the presence of food allergens, either as ingredients or adventitious contaminants. Although the addition of an allergenic food as an ingredient must be always referred in the label, this is not necessarily the case when its presence results from a contamination event (e.g. shared production lines). Contamination events are usually accidental and not likely to occur systematically, implicating that it can happens at varying levels and with heterogeneous distribution, which led to the use of precautionary labelling by food industries. The excessive precautionary labelling was implemented to safeguard the food-allergic consumers and to protect food industry from potential legal problems. However, it represents a very restrictive measure for the allergic individuals since their commercial choices about the available pre-packaged foods are most frequently reduced. In this context, not only the consumers and the food industry, but other stakeholder groups must be committed in managing food allergens across the food supply chain. These include national and international risk managers and authorities involved in setting and enforcing regulations, standardisation and validation bodies, as well as those seeking to provide reliable tools for allergen detection in food [27].

The current approach used by food industry to manage allergens encompasses the existing good manufacturing practices (GMP), within the classic Hazard Analysis Critical Control Points (HACCP) methodology [28], including traceability along the supply chain and discrimination of allergenic ingredients to assure the production of accurately labelled safe food. Despite these stringent measures, industry still faces several difficulties in managing food allergens, thus indicating the need for other alternative actions [29]. Consequently, the food industry and the regulatory authorities have to rely on the available analytical methods to determine the amount of a particular allergic ingredient in a food in order to decide about the safety of the product [26].

Thus, the scientific community is challenged to develop highly precise and accurate analytical techniques for the detection of all the allergenic foods that are currently listed in legislation. Considering that some of the allergenic ingredients relate to large groups of animal and plant species, the challenge faced by researchers is increasingly higher. The ability of one method to detect a specific allergenic ingredient does not necessary imply a similar performance for different species from the same group of allergenic foods [22]. Presently, most of the methods were developed to detect markers of single species of allergenic foods. This evidences that many techniques should be advanced to reliably target all allergenic ingredients with mandatory labelling. In this context, the methods capable of simultaneously detecting multiple allergenic ingredients are starting to be faced as potential answers for these particular issues [22].

Food allergen detection: Proteins or DNA?

In general, the analysis of allergens in foods has been based in two analytical methodologies: the immunoassays that use antibodies raised to recognise specific allergens or protein fractions from allergenic foods and the polymerase chain reaction

(PCR) methods, which detect the presence of DNA from allergenic ingredients. More recently, a third technique has been successfully exploited to trace allergens in foods by means of quantitative methods using mass spectrometry (MS) [30].

The protein-based methods have been referred by some authors as the optimal choices for tracing allergenic ingredients in foods. Due to several advantages such as rapid performance, good specificity, low cost of analysis and commercial availability, the immunoassays namely the enzyme-linked immunosorbent assays (ELISA), have been extensively used for the detection of almost all allergenic foods [31-33]. ELISA are considered the tests that present the lowest limits of detection (LOD) and quantification (LOQ). However, since these assays are based on the biological recognition of the allergen/marker protein via an antibody, they are more prone to cross-react with different food species, giving false positive results. The high susceptibility of proteins of suffering degradation or at least denaturation when submitted to harsh processing conditions (heat treatment, pH alteration, partial hydrolysis, Maillard reactions) such as those usually used to cook foods is another drawback of immunoassays [34-37]. The structural modification of proteins can induce a lost in the ability of the antibodies to identify them, with consequent false negative results.

To overcome the major drawbacks attributed to immunoassays, the methods based on the detection of DNA encoding allergens or other species-specific markers have also been exploited in the past years. DNA is considered a very stable molecule, preserving its integrity even after submitted to severe food processing conditions. This fact has contributed to the recent development of analytical methods based on the detection of DNA over the detection of proteins. Quantitative real-time PCR systems have been successfully applied to several allergenic ingredients with mandatory labelling [31-33], thus providing quantitative information regarding those. When compared to immunoassays, real-time PCR methods present increased specificity because they unlike to cross-react with other food species. Therefore, real-time PCR systems have demonstrated to be excellent alternatives to the use of immunoassays for the detection and quantification of food allergens. The advantages attributed to PCR methods namely relative rapid performance, high level of sensitivity and specificity and moderate cost per analysis have prompted their development and application in the control of food allergens. However, in the opinion of some researchers, the use of methods targeting DNA bears a major disadvantage because of being based on the indirect detection of food allergens. Considering that the correlation between the DNA and the presence of allergenic protein in processed foods might not be constant, there are some factors such as the expression of the allergen being affected by environmental conditions, effects of food processing on DNA and proteins, matrix effects and interference with other compounds and the type of protein fractions/isolates used in product formulation that may lack DNA, cannot be disregarded in this correlation [37]. Another issue of DNA-based methods regards their sensitivity compared to immunoassays, which is generally lower and justifies the need of further improvements.

Included in the protein-based methods, but independent from the typical biological interaction (allergen-antibody), the MS technology has been recently used to target multiple allergens in a single analysis. This technique seems to present several advantages such as the unequivocal identification of allergenic proteins, high levels of sensitivity and simultaneous multitarget analysis. However, the high equipment and maintenance costs and the need for specialised personnel restrain its full application to food allergen detection [38, 39].

Although the number of analytical methods available for allergen detection is increasing rapidly, there is still much research to be performed. In the specific case of the presence of tree nut allergens in foods, that are known to be responsible every year for a great number of the anaphylactic shocks, the need for more accurate and highly specific methods is of utmost importance. Since low quantities of tree nuts are sufficient to induce potentially life-threatening allergic reactions, the development of analytical techniques capable of detecting trace amounts of tree nuts, reaching absolute quantifications at picogramme level and relative quantifications of 1 mg/kg is much needed.

Still, the lack of harmonisation regarding the most suitable methodology (targeting DNA versus proteins) to verify labelling compliance and the absence of available testing/reference materials continues to contribute to the generalised controversy among researchers and represent key issues in the management of food allergens. It is featured that general opinions could come to a compromise, helping to conciliate the most appropriate set of methods for food allergen analysis. In addition, reference materials should also become available to support the development and validation of methods to detect and adequately quantify allergens.

OBJECTIVES AND ORGANISATION OF THE THESIS

In the last years, food allergies have been regarded as an increasing problem of public health. In addition, the growing awareness concerning the quality of life of the sensitised/allergic individuals has led to the implementation of several guidelines aiming at protecting these patients. Since the only means of preventing an allergic reaction consists on the total avoidance of the allergenic food, patients are strongly advised to carefully evaluate the food labelling when choosing processed foods that are commercially available. Although current EU legislations oblige the mandatory labelling of potentially allergenic foods by food industry, the sensitised/allergic individuals still face some uncertainties. If by one side, labels can contain excessive precautionary information, on the other hand they can miss crucial evidence of potentially hidden allergens as result of cross-contaminations during food preparation. To help food industry complying with labelling and to provide regulatory authorities with efficient tools for the management of food allergens, the development of highly sensitive and accurate techniques is of extreme importance.

In this context, the main objectives of this work regarded:

- The development of novel molecular methodologies, mostly based on real-time PCR, for the detection and quantification of tree nuts in foods;
- Application of protein-based methods ELISA, immunoblotting and Liquid Chromatography – Mass Spectrometry (LC-MS/MS) for the detection and quantification of tree nut allergens;
- Comparison of DNA-based techniques with protein-based methods.

Additionally, other specific topics were considered of high relevance along the performance of this study:

- The preparation of adequate model mixtures for the development and validation of the proposed techniques, since no reference or testing materials are available for allergen analysis;
- The evaluation of food processing (e.g. heat treatment) on the performance of the novel real-time PCR methods;
- The discrimination of genetically related species (e.g. almond, cherry, peach or apricot) using the high resolution melting (HRM) analysis;
- The development of improved sensitivity and innovative real-time PCR methods having in consideration the actual “MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments” [40].

According to the proposed objectives and to better integrate and correlate all the contents that were addressed during the course of this PhD, the thesis was organised in three main chapters that come out after a General Introduction. An overview on the definitions on food allergies and food allergens, the prevalence and management of food allergies was presented, followed by brief state-of-the art methodologies to detect food allergens, in which the need of further research was emphasised. The main focus of this work was on the selected three tree nuts, namely almond, hazelnut and walnut, because of their frequent consumption, significant clinical relevance and the adequacy of DNA-based methods to their detection.

Chapter 1 regards almond as an allergenic food, being composed by a state-of-the-art section (review) describing the most important topics related to this nut. The experimental section is composed by three papers concerning the development of novel DNA-based methods to target almond in processed foods (e.g. chocolates, cakes).

Chapter 2 is dedicated to the study of hazelnut as a food allergen. In this chapter, it is provided an overview about the major relevant issues associated with hazelnut allergy (review paper). In the experimental part, three papers are presented relating the development and comparison of different protein- and DNA-based methods for hazelnut detection.

Chapter 3 concerns to the last nut studied during the course of this work. Like the two previous chapters, the first section presents a state-of-the-art about walnut as a food allergen (review). The experimental section reports in one paper regarding the development of a novel DNA-based technique to trace walnut in cakes as affected by food processing.

The concluding remarks of this work and future trends, highlighting the major achievements for each of the studied tree nut are presented as a final section.

This thesis is the outcome from the research work mostly developed in the Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto. A part of the research was performed in the Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants, Center of Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Austria.

BIBLIOGRAPHY

(1) Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. J Allergy Clin Immunol 2010; 126(6): S1-S58.

(2) Madsen CB, Hattersley S, Allen KJ, Beyer K, Chan CH, Godefroy SB et al. Can we define a tolerable level of risk in food allergy? Report from a EuroPrevall/UK Food Standards Agency workshop. Clin Exp Allergy 2012; 42(1): 30-37.

(3) EAACI. Food allergy and anaphylaxis public declaration. European Academy of Allergy and Clinical Immunology (EAACI). Available at: http://www.eaaci.org/attachments/FoodAllergy&AnaphylaxisPublicDeclaration.pdf (Accession on 15/11/2013)

(4) Stanley JS, Bannon GA. Biochemistry of food allergens. Clin Rev Allergy Immunol 1999; 17(3): 279-291.

(5) Steckelbroeck S, Ballmer-Weber BK, Vieths S. Potential, pitfalls, and prospects of food allergy diagnostics with recombinant allergens or synthetic sequential epitopes. J Allergy Clin Immunol 2008; 121(6): 1323-1330.

(6) Breiteneder H, Radauer C. A classification of plant food allergens. J Allergy Clin Immunol 2004; 113(5): 821-830.

(7) Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. J Allergy Clin Immunol 2000; 106(1): 27-36.

(8) Sicherer SH, Sampson HA. Food allergy. J Allergy Clin Immunol 2006; 117(2, Supplement 2): S470-S475.

(9) Costa J, Oliveira MBPP, Mafra I. Alergénios alimentares: o que são, o que provocam e como detetá-los? Química 2012; 127: 33-38.

(10) Sicherer SH. Food Allergy. Mt Sinai J Med 2011; 78(5): 683-696.

(11) Chafen JJS, Newberry SJ, Riedl MA, Bravata DM, Maglione M, Suttorp MJ, et al.. Diagnosing and managing common food allergies: a systematic review. JAMA 2010; 303(18): 1848-1856.

(12) Sicherer SH, Sampson HA. Food Allergy: Recent Advances in Pathophysiology and Treatment. Annu Rev Med 2009; 60(1): 261-277.

(13) Costa J, Mafra I, Carrapatoso I, Oliveira MBPP. Hazelnut allergens: molecular characterization, detection, and clinical relevance. Crit Rev Food Sci Nutr (accepted).

(14) Mills ENC, Mackie AR, Burney P, Beyer K, Frewer L, Madsen C, et al. The prevalence, cost and basis of food allergy across Europe. Allergy 2007; 62(7): 717-722.

(15) Shah E., Pongracic J. Food-induced anaphylaxis: who, what, why, and where? Pediatr Annu 2008; 37(8): 536-541.

(16) Sicherer SH. Epidemiology of food allergy. J Allergy Clin Immunol 2011; 127(3): 594-602.

(17) Eller E, Hansen TK, Bindslev-Jensen C. Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. Ann Allergy Asthma Immunol 2012; 108(5): 332-336.

(18) Blom WM, Vlieg-Boerstra BJ, Kruizinga AG, van der Heide S, Houben GF, Dubois AEJ. Threshold dose distributions for 5 major allergenic foods in children. J Allergy Clin Immunol 2013; 131(1): 172-179.

(19) Costa J, Mafra I, Carrapatoso I, Oliveira MBPP. Almond allergens: molecular characterization, detection, and clinical relevance. J Agric Food Chem 2012; 60(6): 1337-1349.

(20) Berin MC, Sampson HA. Food allergy: an enigmatic epidemic. Trends Immunol 2013; 34(8): 390-397.

(21) Nguyen-Luu NU, Ben-Shoshan M, Alizadehfar R, Joseph L, Harada L, Allen M, et al. Inadvertent exposures in children with peanut allergy. Pediatr Allergy Immunol 2012; 23(2): 134-140.

(22) van Hengel AJ. Introduction. In: Nollet LML, van Hengel A.J. editors. Food allergens: analysis instrumentation and methods. Boca Raton: CRC Press, Taylor & Francis Group; 2011. pp. 1-11.

(23) Tang MLK, Martino DJ. Oral immunotherapy and tolerance induction in childhood. Pediatr. Allergy Immunol 2013; 24(6): 512-520.

(24) Commission of the European Communities, Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC regarding certain food ingredients. Off. J. Eur. Union. L310, 11-14.

(25) The European Parliament and the Council of the European Union, Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. Off. J. Eur. Union. L304, 18-63.

(26) Cucu T, Jacxsens L, De Meulenaer B. Analysis to support allergen risk management: which way to go? J Agric Food Chem 2013; 61(24): 5624-5633.

(27) Kerbach S, Alldrick AJ, Crevel RWR., Domotor L, DunnGalvin A, Mills ENC et al. Managing food allergens in the food supply chain - viewed from different stakeholder perspectives. Qual Assur Saf Crop Foods 2009; 1(1): 50-60.

(28) Codex Alimentarius Commission. General Principles of Food Hygiene. Hazard Analysis and Critical Control Point (HACCP) system and guidelines for its application, Annex to CAC/RCP 1-1969 (Rev. 4 - 2003) Available at: http://www.codexalimentarius.org/standards/list-of-standards/en/?no_cache=1 (last accessed 24/10/2013)

(29) Ward R, Crevel R, Bell I, Khandke N, Ramsay C, Paine S. A vision for allergen management best practice in the food industry. Trends Food Sci Technol 2010; 21(12): 619-625.

(30) Johnson PE, Sancho AI, Crevel RWR, Mills ENC. Detection of allergens in foods. In: Nollet LML, van Hengel A.J. editors. Food allergens: analysis instrumentation and methods. Boca Raton: CRC Press, Taylor & Francis Group; 2011. pp. 13-27.

(31) Nollet LML, van Hengel AJ, editors. Food allergens: analysis instrumentation and methods. Boca Raton: CRC Press, Taylor & Francis Group; 2011.

(32) Popping B, Diaz-Amigo C, Hoenicke K, editors. Molecular biological and immunological techniques and applications for food chemists. New Jersey: John Wiley & Sons, Inc.; 2010.

(33) Koppelman SJ, Hefle SL, editors. Detecting allergens in food. Boca Raton: CRC Press, Taylor & Francis Group; 2006.

(34) Mills ENC, Sancho AI, Rigby NM, Jenkins JA, Mackie AR. Impact of food processing on the structural and allergenic properties of food allergens. Mol Nutr Food Res 2009; 53(8): 963-969.

(35) Paschke A. Aspects of food processing and its effect on allergen structure. Mol Nutr Food Res 2009; 53(8): 959-962.

(36) Sathe SK, Sharma GM. Effects of food processing on food allergens. Mol Nutr Food Res 2009; 53(8): 970-978.

(37) Diaz Amigo C., Popping B. Detection of food allergens. In: Popping B, Diaz-Amigo C, Hoenicke K, editors. Molecular biological and immunological techniques and applications for food chemists. New Jersey: John Wiley & Sons, Inc.; 2010. pp. 175-198.

(38) Johnson PE, Baumgartner S, Aldick T, Bessant C, Giosafatto V, Heick J. et al. Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. J AOAC Int 2011; 94(4): 1026-1033.

(39) Monaci L, Visconti A. Mass spectrometry-based proteomics methods for analysis of food allergens. Trac-Trends Anal Chem 2009; 28(5): 581-591.

(40) Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009; 55(4): 611-622.

CHAPTER 1. ALMOND

STATE-OF-THE-ART

Almond allergens: molecular characterization, detection and clinical relevance

Journal of Agricultural and Food Chemistry, **2012**, *60*, 1337-1349

EXPERIMENTAL PART

High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods

Food Chemistry, **2012**, *133*, 1062-1069

Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods

Food Research International, **2013**, *51*, pp 228-235

Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols

Food Chemistry (submitted)

STATE-OF-THE-ART

Almond allergens: molecular characterization, detection and clinical relevance

Journal of Agricultural and Food Chemistry, **2012**, *60*, 1337-1349

ACS Publications © 2012 American Chemical Society

J. Agric.Food Chem. 2012, 60, 1337−1349

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

pubs.acs.org/JAFC

Almond allergens: molecular characterization, detection and clinical relevance

Joana Costa†, Isabel Mafra*,†, Isabel Carrapatoso‡, Maria Beatriz P.P. Oliveira†

†REQUIMTE, Laboratório de Bromatologia e Hidrologia, Faculdade de Farmácia, Universidade do Porto, Portugal, ‡Serviço de Imunoalergologia. Hospitais da Universidade de Coimbra, Portugal.

*Corresponding author: Tel: +351 222078902. Fax: +351 222003977. E-mail: isabel.mafra@ff.up.pt

ABSTRACT:

Almond (Prunus dulcis) has been widely used in all sorts of food products (bakery, pastry, snacks) mostly due to its pleasant flavor and to its health benefits. However, it is also classified as a potential allergenic seed known to be responsible for triggering several mild to life-threatening immune reactions in sensitized and allergic individuals. Presently, eight groups of allergenic proteins have been identified and characterized in almond, namely PR-10 (Pru du 1), TLP (Pru du 2), prolamins (Pru du 2S albumin, Pru du 3), profilins (Pru du 4), 60sRP (Pru du 5) and cupin (Pru du 6, Pru du γ-conglutin), although only few of them have been tested for reactivity with almond-allergic sera. To protect sensitized individuals, labeling regulations have been implemented for foods containing potential allergenic ingredients, impelling the development of adequate analytical methods. This work aims to present an updated and critical overview about the molecular characterization and clinical relevance of almond allergens, as well as revising the main methodologies used to detect and quantitate food allergens with special emphasis to almond.

Keywords: Food allergy, Almond, Tree nuts, Immunological reactions, Allergen detection

INTRODUCTION

Tree nuts have attained an important place in human diets since they are considered excellent foods, mainly due to their pleasant taste and potential health benefits. They are consumed all over the world by the majority of individuals in a wide variety of forms, which are more or less related to the population habits and/or to the type of tree available in the geographical region. For instance, in 2009 in Portugal, 33% of the total tree nut production corresponded to almond fruits and 5% of the annual fruit consumption was from tree nuts.[1] Almond (*Prunus dulcis* or *Amygdalus communis* L.) is one of the most commonly consumed nuts, together with hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoiesis*), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia nuts (*Macadamia ternifolia*) and pine nuts (*Pinus pinea* and other *Pinus* species). Among those, almond occupied the first place in terms of global trade in 2009, followed by cashew, pistachio and hazelnut.[2] Considering the world production of tree nuts in 2010, almond ranked the third position on the global basis, after cashew and walnut productions, with USA and Spain as the two major producers of almond.[2]

In Europe, tree nuts such as almond are far more consumed than peanuts or seeds.[3] As a consequence, tree nuts have occupied an important place in the economy since they are an integral part of the human food supply. Tree nuts can be consumed either raw (snacks) or processed, being its edible fraction used as ingredient in a wide variety of food products (spreads, bakery, pastry, chocolates and confectionary products).[3] The increasing consumption of tree nuts has been related to the potential health benefits of these foods. With the present recognition by the Food and Drug Administration (FDA) regarding the health benefits attributed to tree nuts, namely as "heart-protective" foods, the consumption of these nuts has risen mainly in developed countries.[4]

However, in recent years, the use of tree nuts in food has also led to concerns about the growing number of sensitized individuals to tree nuts and peanuts, especially in western countries (Europe and USA).[5] In USA, by the use of random-calling telephone surveys, through a 11-year follow up study, there was an increase of tree nut allergy prevalence in children, ranging from 0.6% in 1997, to 1.2% in 2002 and 2.1% in 2008, whereas in adult population the same prevalence remained around 1.3%.[6] In Europe, hazelnut allergy is common and often associated with birch pollinosis, while in USA allergy to walnut, cashew, almond, pecan and Brazil nut appears to be more common than hazelnut.[7] Nevertheless, recent data from Europe, USA and Australia identified hazelnut as the food with the highest sensitization rate.[8]

 Journal of Agricultural and Food Chemistry, **2012**, *60*, 1337-1349

In 1985, the Codex Alimentarius Commission first listed a set of food products in which tree nuts were part, as likely to cause hypersensitivity in sensitized individuals, advising the obligation to label foodstuffs containing possible allergens.[9] In 1993, the same Commission included the tree nuts in the group of eight foods known to be responsible for almost 90% of human food allergies. Since then, special attention has been devoted to establish clear guidelines for food allergen labeling, compelling the European Union (EU) to first include the allergenic foods in the Directive 2000/13/EC.[10] Accordingly, the producers have the obligation to declare all ingredients present in pre-packaged foods traded inside the EU, with very few exceptions. The Directive 2000/13/EC has been updated several times with new amendments concerning the list of potential allergens.[10] The two most important amendments were the Directive 2003/89/EC Annex IIIa[11] and the Directive 2007/68/EC[12]. The former included a list of 12 allergenic foods (cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame and sulphur dioxide) that must always be declared on the label of foodstuffs.[11] The latter amendment lists the 14 allergenic foods (including two more foods, namely lupine and mollusks), as well as some exemptions that are not required to be labeled.[12]

This review intends to provide an updated and critical overview on almond allergens, regarding their biochemical and biological characterization, as well as clinical implications on sensitized individuals. It also aims to assemble the techniques, focusing on the recent developments on protein and DNA-based methods for monitoring the presence of almond allergens in food products in compliance with the labeling statements.

FOOD ALLERGY

Food allergies can occur upon the ingestion of allergenic food components that in sensitized individuals can trigger mild to severe abnormal responses mediated by the immunological system. The adverse response to food proteins (mainly glycoproteins) can be mediated by the immunoglobulin-E (IgE) or non-IgE (cellular) mechanisms and are estimated to affect almost 3-4% of adults and 6% of young children.[13] For reasons not yet well understood, tree nuts pose serious health problems to sensitized individuals, which frequently come across with negative physiological responses and can vary in intensity upon exposure to these seeds.[5] Tree nuts are known to be responsible for triggering abnormal immunological responses in allergic individuals, ranging from mild reactions to potentially fatal anaphylactic shocks. More than one third of total anaphylactic reactions that occurred in western countries are thought to be provoked by food ingestion and are often attributed to peanut, tree nuts or shellfish ingestion.[14] According to Sicherer[15], there are no studies to address directly the prevalence of fatal food-allergic reactions. Fatalities have been mainly reported from allergic reactions to peanuts and tree nuts, appearing to be associated with delayed treatment with epinephrine, and occuring more often in teenagers and young adults with asthma and previously diagnosed food allergy. In a population-based USA registry, 31 deaths were registered from 2001 to 2006, in which 6 were caused by tree nuts.[16] Pumphrey and Gowland[17] reported 48 deaths in United Kingdom from 1999 to 2006, in which 9 were related with tree nuts, ascertain all food-related anaphylaxis.

Hypersensitivity reactions are catalogued into four groups, according to the mechanism responsible for the immunological response. Type I-hypersensitivity reactions are IgE-mediated through the activation of the mast cells, type II and III are IgG-mediated and type IV are triggered by T_H1 and T_H2 cells.[5] Food allergies are essentially included in type I category due to the specific IgE antibodies production against food allergens. In this case, the allergic reactions result from a previous sensitization to the allergen, generally leading to the release of histamine and other biological mediators in subsequent exposures.[5]

Allergic reactions related to food ingestion can appear within minutes up to 2 hours after the ingestion, involving one or several target organs like the skin, the gastrointestinal and respiratory tracts as well as the cardiovascular system.[18] The most severe allergic manifestation is anaphylaxis, that can be fatal or near fatal, even when only traces of the allergen are ingested. Tree nuts, such as almond, are among the food products related to this type of reactions.[14] Other less serious responses such as cutaneous reactions are the most common clinical manifestation of food allergy and are frequently observed in combination with symptoms of other target organs. The oral allergy syndrome (OAS) is another clinical manifestation associated to food allergies and generally appears within 5 to 15 minutes after the allergen ingestion. Fresh vegetables, fruits and tree nuts are typically the responsible agents for this type of reactions.[18]

ALMOND ALLERGENS

Almond is taxonomically designated as *Prunus dulcis* or *Amygdalus communis* L. (the most common denominations) and belongs to the Rosaceae family, subfamily of the Prunoideae. Rosaceae family also includes fruits such as peach, apricot, plum, cherry (Prunoideae subfamily), apple, pear (Pomoideae subfamily), blackberry and strawberry (Rosoideae subfamily).[19-21]

Almond allergy in the third most commonly reported tree nut allergy in the USA (reactive in 15% of patients), behind cashew nut and walnut.[22] Some native allergens have been identified and characterized according to their biochemical function, although only few have been cloned or tested for their reactivity with sera from almond-allergic patients.[23] Until now, eight groups of allergens were identified in almond: Pru du 1, Pru du

2, Pru du 2S albumin, Pru du 3, Pru du 4, Pru du 5, Pru du 6 (amandin) and Pru du γ-conglutin. From these eight groups: Pru du 3, Pru du 4, Pru du 5 and Pru du 6 are recognized and included in WHO-IUIS list of allergens.[24] Their biochemical designations, clinical relevance, biological functions and accession numbers from NCBI[25] Database are summarized in **Table 1**.

Pru du 1 (PR-proteins). One group of allergens identified in almond that comprises a family of proteins named as Pru du 1, included in Group 1 Fagales-related protein, most commonly known as Pathogenesis Related-10 proteins (PR-10) (**Table 1**). The PR-10 family encompasses a particular set of proteins that are related to intracellular defense mechanisms and response to fungal or bacterial infections. PR-10 proteins exist in various isoforms, differing in their IgE-binding capacities.[26] The Bet v 1-homologous food allergens are thought to function as plant steroid hormone transporters,[27] and they have been identified in several Rosaceae fruits, including almond.[19,26] Bet v 1-homologues are commonly labile proteins and in general can suffer unfolding during the cooking process. The boiling process (wet processing) causes the destruction of the conformational epitopes, reducing the IgE-reactivity and their ability to trigger allergic reactions in sensitized individuals.[28] In almond, seven members of PR-10 proteins have been identified. The genes encoding the putative isoallergens Pru du 1.01 to Pru du 1.06A/B have already been cloned and mapped.[25] Amino acid sequences of Pru du 1.01 to Pru du 1.05 possess more than 5% of dissimilarity among these five proteins, placing them into different isoallergen groups. Pru du 1.06A and Pru du 1.06B present over 95% of DNA sequence identity, putting these two proteins within the same group of isoallergens. All seven proteins possess peptide sequences of 160 amino acids (aa), with the exception of Pru du 1.04 that contains 159 aa. The predicted molecular weight of the described proteins ranges between 17.1-17.5 kDa with isoelectric point (pI) values varying from 4.9 to 6.0.[19] PR-10 proteins from almond are also very similar to those found in apple (Mal d 1),[29] pear (Pyr c 1),[30] sweet cherry (Pru av 1)[31] and apricot (Pru ar 1).[32]

Pru du 2 (TLP). Pru du 2 is group of allergens identified in almond with five putative isoallergen genes (*Pru du 2.01A/B* to *Pru du 2.04*) already cloned and sequenced (**Table 1**).[19,25] These allergens belong to the PR-5 family, also known as thaumatin-like proteins (TLP), comprising three groups of responses: to pathogen infection, to osmotic stress (osmatins) and to fungal proteins.[33] The TLP identified in almond possess protein weights ranging from 23 to 27 kDa and different sequence sizes. Pru du 2.01A/B and Pru du 2.02 contain 246 aa, while Pru du 2.03 and Pru du 2.04 contain 277 and 330 aa, respectively. Like PR-10 proteins, the TLP comprise four different isoallergen groups, displaying a signal peptide of 24 aa, with the exception of Pru du 2.02 protein that has in its signaling sequence 21 aa.[19]

Table 1. Identification of almond allergens according to their biological function, clinical relevance and respective accession numbers in the NCBI database.[25]

Name	Biochemical designation	Protein Families	Molecular Weight (kDa)	Biological Function	Clinical relevance	Isoallergen designation	Isoforms or variants	Nucleotide	Protein
Pru du 1	Bet v 1-homologous	PR-10 family	17 (~160 aa)	Protection against pathogenic constraints and adaptation to stressful environment	Mild immune reactions and related to OAS. Severe allergic reactions reported in some patients with birch pollen allergy. Cross-reactivity with Bet v 1 and other PR-10.	Pru du 1.01 Pru du 1.02 Pru du 1.03 Pru du 1.04 Pru du 1.05 Pru du 1.06A Pru du 1.06B	Not known	EU424239.1 EU424241.1 EU424243.1 EU424245.1 EU424247.1 EU424251.1 EU424249.1	ACE80939.1 ACE80941.1 ACE80943.1 ACE80945.1 ACE80947.1 ACE80951.1 ACE80949.1
Pru du 2	TLP	PR-5 family	23–27 (246 aa) (246 aa) (246 aa) (277 aa) (330 aa)	Thaumatins	Recognized as potent allergens but the clinical relevance is yet subject of study.	Pru du 2.01A Pru du 2.01B Pru du 2.02 Pru du 2.03 Pru du 2.04	Not known	EU424256.1 EU424258.1 EU424254.1 EU424260.1 EU424262.1	ACE80956.1 ACE80958.1 ACE80954.1 ACE80960.1 ACE80962.1
Pru du 2S albumin	2S Albumin	Members of the prolamin superfamily	12 (28 aa)	Seed storage proteins for seed development	Specific allergic symptoms not yet defined. More studies needed.	Pru du 2S albumin	Not known	Not known	P82944.1
Pru du 3	nsLTP	Members of prolamin superfamily	9 (117 aa) (123 aa) (116 aa)	Lipid transfer proteins	Systemic and life-threatening symptoms. Cross-reactivity among Rosaceae fruits	Pru du 3.01 Pru du 3.02 Pru du 3.03	Pru du 3.0101	EU424264.1 EU424266.1 EU424268.1	ACE80964.1 ACE80966.1 ACE80968.1
Pru du 4	Profilin	Profilin-specific IgE usually cross-reacts with homologues from virtually every plant source	14 (131 aa)	Actin-binding proteins	Symptoms are mild and limited to oral cavity.	Pru du 4.01 Pru du 4.02	Pru du 4.0101 Pru du 4.0102	EU424270.1 EU424272.1	ACE80970.1 ACE80972.1
Pru du 5	r60sRP	Autoimmune reactions to human P2	10 (113 aa)	Intervenes in the elongation step of protein synthesis	Specific allergic symptoms not yet defined. More studies needed.	Pru du 5	Pru du 5.0101	AY081851.1	AAL91663.1
Pru du 6	Amandin, 11S globulin, or AMP	Members of cupin superfamily	360 (~1055 aa)	Legumin-like protein (major storage protein)	Reported to induce severe allergic reactions.	Prunin-1 Prunin-2	Pru du 6.0101 Pru du 6.0201	X78119.1 X78120.1	CAA55009.1 CAA55010.1
Pru du γ-conglutin	γ-Conglutin	Members of cupin superfamily	45 (25 aa)	7S Vicilins	Specific allergic symptoms not yet defined. More studies needed.	Not known	Not known	Not known	P82952.1

The TLP are very resistant to proteases, pH or heat-induced denaturation, probably due to the presence of sixteen conserved cysteine residues bonded in eight disulphide bridges.[33] These biochemical characteristics are most likely the reason why these proteins can affect sensitized individuals, since they are not significantly destroyed by the usual food processing methods.

Pru du 2S albumin. The 2S albumins are included in the prolamin superfamily (**Table 1**). This group encompasses other allergenic proteins such as the non-specific lipid-transfer proteins (nsLTP), the α-amylase/trypsin inhibitors and the prolamin storage proteins.[34] The 2S albumins act as seed storage proteins for seed developing and as defense-related proteins.[35] They are thought to cause sensitization along the gastrointestinal tract, suggesting that 2S albumins are resistant, at least to some extent, to adverse conditions such as acidic pH, proteolytic activity of digestive enzymes and denaturing effects of surfactants.[36] The secondary structure of the 2S albumin seems to remain unaltered below 90 ºC,[28] preserving their allergenic capacity when exposed to the immune system and, therefore, inducing allergic responses in sensitized individuals.[36] 2S Albumins present high structural homology. However, cross-reactivity between allergens with less than 50% amino acid sequence homology is rare. Cross-reaction typically requires an amino acid sequence homology of more than 70%.[37] 2S Albumin protein identified in almond is classified as a major allergen.[36,38] Pru du 2S albumin has a peptide sequence of 28 aa and a molecular weight of 12 kDa, preserving a fraction of 6 kDa after enzyme digestion, which maintains IgE-binding activity. A second fraction of 2 kDa, belonging to the 2S albumin was also sequenced, revealing 80% of similarity with the sequences near the C-terminal of English walnut (allergen Jug r 1) and Brazil nut 2S albumin. This fact, along with the high content in methionine of the 6 kDa fraction, suggests that this protein is possibly a member of the 2S albumin allergen family.[38] Although Pru du 2S albumin exhibits over 80% of similarity with Brazil nut 2S albumin, no cross-reactivity has been suggested to occur between these two nuts.[39] Besides high sequence homology, shared linear epitopes among 2S albumins are apparently linked to cross-reactivity.[36]

Pru du 3 (nsLTP). Like the 2S albumins, the allergenic nsLTP belong to the prolamin superfamily, being also known as the PR-14. In almond, three nsLTP (Pru du 3) were identified and characterized.[19] The genes encoding Pru du 3.01 to 3.03 proteins were sequenced and made available at NCBI database (**Table 1**). The Pru du 3.01, 3.02 and 3.03 isoallergens have similar molecular weights (9 kDa), belong to the nsLTP type 1 subfamily, but have different sizes: 117, 123 and 116 aa with distinct signal sequences of 26, 30 and 25 aa, respectively.[25] The three isoallergens exhibit eight conserved cysteine residues,[19] enabling the conformation of four disulphide bridges. Like other plant nsLTP, this subfamily of nsLTP type 1 includes small and soluble proteins to facilitate the transference of lipids (fatty acids, phospholipids, glycolipids and steroids) between membranes. nsLTP possess an internal hydrophobic core that functions as the binding site for lipids. Besides lipid transport and assembly, they also intervene in the defense of plants against fungal and bacterial activities.[25,40] Many nsLTP1 proteins such as the case of Pru du 3 (Pru du 3.01 to 3.03) have been characterized as allergens in humans.[25]

Since nsLTP are usually accumulated in the outer epidermal layers of plant organs, they are thought to be responsible for the stronger allergenicity of the peels in comparison to the inner layers of the fruit (pulps) in the Rosaceae family. These proteins are also very resistant to abrupt pH changes, thermal treatments, and pepsin digestion, having the ability to refold to their functional structures after cooling. Belonging to the same prolamin superfamily, nsLTP are only slightly less thermally stable than the 2S albumins, possibly due to the presence of a lipid binding tunnel.[28] This group of molecules is included in the so-called panallergens that are, by definition, allergens ubiquitously spread throughout nature. Although the molecules are originated from different and unrelated organisms, they are composed by similar conserved sequence regions. nsLTP family presents highly conserved sequences and tridimensional structures that enable IgE recognition, promoting cross-reactivity among these type of proteins.[40] In addition to these facts, nsLTP are present in diverse Rosaceae fruits and seeds such as apple, peach, plum, cherry sweet, apricot, and almond, implicating a probable cross-reactivity among them.[26]

Pru du 4 (Profilins). Pru du 4 proteins belong to the profilin family and are encoded by the putative allergen genes *Pru du 4.01* and *Pru du 4.02* (**Table 1**).[23,25] *Pru du 4.01* and *4.02* genes exhibit fragments of different sizes, 1041 and 754 base pairs (bp), respectively, encoding two proteins with identical sequences (131 aa), molecular weight of approximately 14 kDa and acidic properties (pI approximately of 4.6). Profilins participate in the binding of a monomeric actin (G-actin) that is responsible for establishing a high-affinity complex with actin, regulating the polymerization of actin into filaments.[41] Like the nsLTP, profilins are also classified as panallergens. These proteins display a high degree of similarity and identity with several other profilins from diverse plant and tree species, revealing cross-reactivity due to the highly preserved amino acid sequences as well as to the shared IgE-reactive epitopes.[23,42] IgE cross-reactivity is related to the general three-dimensional profilin fold, being composed of five stranded anti-parallel β-sheet and two α-helices.[43] According to Tawde et al.,[23] allergens Pru p 4.01 and Pru av 4 from peach and sweet cherry, respectively, are the two proteins presenting the highest identity and similarity (99% and 98%) with almond profilins. Even apparently non-related species such as soybean (*Glycine max*) or olive (*Olea europeae*) exhibit more than 80% of identity and 90% of similarity with Pru du 4 allergen.[23] Therefore, it is not unexpected that a profilin from one plant species can cross-sensitize an individual to other plant tissues, such as pollen profilins can sensitize individuals to food profilins.[44] The sensitization to these proteins can result in allergic reactions to proteins from a wide range of fruits and vegetables, including fruits or seeds of the Rosaceae family such as Mal d 4 in apple, Pru p 4 in peach, Pru av 4 in sweet cherry or Pru du 4 in almond, among many others.[23,45]

Unlike other food allergens such as nsLTP or 2S albumins, profilins seem to display moderate structural stability,[23] since adverse conditions contribute to the denaturation of profilins and subsequent loss of conformational structure. The labile character of Pru du 4 profilin and the low levels of this protein in almond explain the difficulty for its detection by immunoblot screens. Profilins are generally defined as minor but rather important allergens in many plant foods. The positive detection of almond profilin in 44% of the patients' sera suggests the classification of Pru du 4 as a minor but important allergen.[23]

Pru du 5 (60s acidic ribosomal protein P2). Almond allergen Pru du 5, also known as 60S acidic ribosomal protein P2, is encoded by the *Prunus dulcis* 60S acidic ribosomal protein gene (*AL60SRP*), with a size of 604 bp (**Table 1**).[24,25] The ribosomal P2 proteins occur in the ribosome as multimers appearing as sets of heterodimers. P2 proteins seem to be more externally located and subsequently more likely to interact with other cellular components. The biological function of this protein is based on the successive addition of amino acid residues to a polypeptide chain during protein biosynthesis.[25] The expression of a recombinant 60S ribosomal protein of almond (r60sRP) enabled to calculate a molecular mass of approximately 11.4 kDa with a deduced peptide sequence of 113 aa, being reported for the first time in 2009 as an almond allergen.[46] This protein exhibits 81% of identity and 94% of homology with the recently described protein ARP60S from tomato,[47] which may indicate possible cross-reactivity between them. The presence of IgE antibodies for r60sRP in 50% of sera of sensitized patients' to almond seems to classify this protein as a major allergen in almond,[46] according to the allergen nomenclature guidelines specified by the International Union of Immunological Societies (I.U.I.S.) Allergen Nomenclature Sub-committee.[24] Nonetheless, this classification must be supported with more studies regarding the IgE reactivity of patients' sera to this allergen.

Pru du 6 (Amandin). Amandin or almond major protein (AMP) is normally referred to be a member of the cupin superfamily, specifically belonging to the 11S seed storage globulin family.[26,48] The globulins are highly abundant proteins, accounting for more than 50% of the total seed proteins in various legumes and tree nuts. The globulins are divided in two groups, namely, the 7S vicilin-type, and the 11S legumin-type in which the amandin protein is included (**Table 1**). The functional 11S-legumins are hexameric proteins, comprising six subunits with a total molecular weight of about 360 kDa.[26] Isolation and sequencing of cDNA clones from almond enabled to infer that the cDNA encoded two seed storage proteins with 61.0 kDa and 55.9 kDa, designated as prunin-1 (Pru-1) and prunin-2 (Pru-2), respectively (**Table 1**).[49] Both Pru-1 and Pru-2 have two polypeptides linked by disulphide bonds with 551 and 504 aa, respectively. Pru-1 is composed of an acidic α-chain of 40.1 kDa with a pI of 5.4 and a basic β-chain of 20.9 kDa with a pI of 9.6. Pru-2 is divided in two subunits of 34.5 kDa (pI 4.6) and 21.4 kDa (pI 9.5), corresponding to the α- and β-chains, respectively.[49] Pru-1 is highly water soluble and readily cold-precipitable like other proteins from the 11S family and was recently identified as a major component of almond amandin. Pru-1 and Pru-2 are assembled in a functional protein (amandin) by means of disulphide bonds, conferring an elevated thermal stability to the entire protein.[50]

Amandin is classified, both as a major protein component and as a major allergen in almond,[51,52] although the IgE epitopes of Pru-1 or amandin have not yet been identified.[53] 11S Globulins, such as amandin, are thermally stable proteins known to suffer partial unfolding only at temperatures over 94 ºC, aggregating to form different structures within foods. The denaturation process of this type of proteins, which consequently decreases their allergenicity, involves the presence of water. Almonds are often thermally treated at low-water system such is the case of roasting that rather increases the thermal stability of these proteins.[54] Until now, amandin is the most widely studied allergen in almond regarding its molecular structure and biochemical function.[50-53,55]

Pru du γ-conglutin. The γ-conglutin proteins belong to the vicilins (7S globulins) of the cupin superfamily. These proteins have trimeric structures with a molecular weight of approximately 150 to 190 kDa, with each subunit ranging from 40 to 80 kDa (**Table 1**). The composition of each subunit diverges considerably, essentially due to their differences in the extent of post-translational processing (proteolysis and glycosylation).[56] Like in other fruits and seeds where conglutins have been identified and characterized, such as in peanut,[57] soybean,[58] cashew,[59] or lupine,[60] γ-conglutin was also identified in almond with a peptide sequence of 25 aa and a molecular weight of 45 kDa.[38] This protein comprises IgE-binding epitopes located in the 30 kDa *N*-terminal region of the sequence. Since seed conglutins are processed in two subunits, one small C-terminal subunit of 17 kDa and a heavy chain *N*-terminal subunit of 28-30 kDa, it was advanced that the 30 kDa almond peptide corresponded to the heavy chain of the γ-conglutin protein.[38] A sequence identity of about 40% and homology of 60% was found between the mature form of γ-conglutin from white and narrow-leafed blue lupine and γ-conglutin from almond. High similarity (50%) was also observed between the 7S globulin from soybean and the conglutin-like protein from *Arabidopsis*,[38] contributing to a probable cross-reactivity among these seeds.

CLINICAL SYMPTOMS ATTRIBUTED TO ALMOND ALLERGENS

According to the clinical manifestations, the physical/chemical characteristics of plant-derived food allergens and the underlying immunological mechanisms, two different classes of IgE-mediated food allergies can be distinguished. In Class 1, food allergy sensitization occurs through the gastrointestinal tract and is often caused by stable allergens. This class of food allergy is more frequent in children. In contrast, Class 2 food allergy is more likely to appear later in life, affecting mostly adolescents and adults. This allergy is most probably developed as a consequence of sensitization to inhaled allergens. The basis for Class 2 food allergy is immunological cross-reactivity due to high amino acid sequence identity and structural homology between food and inhaled allergens.[61]

Almond allergy is frequently associated with allergies to other fruits from the Rosaceae family in patients sensitized to birch pollen. This pattern of sensitization is more common in Northern European countries in the context of a cross-reactive syndrome to PR-10 proteins, where multiple sensitizations to different pollens, fruits, nuts and other vegetables can occur. In most cases, immunological reactions are typically mild and its prominent clinical manifestation is related to the OAS. However, severe allergic reactions have been attributed to members of the PR-10 protein family in allergic patients to birch pollen.[62,63] This type of reactions arises from the homology among Pru du 1, Bet v 1 and other PR-10 allergens.[32,64,65]

Food allergy related to almond and other Rosaceae fruits can also happen without previous relevant pollen sensitization and is often attributed to allergens from the nsLTP family, in which Pru du 3 is included. The symptoms are frequently systemic and life-threatening, and cross-reactivity among nsLTP of different Rosaceae fruits has been described.[66,67] This pattern of sensitization is more recurrent in Mediterranean countries where fruits from the Rosaceae family are widely cultivated. The nsLTP allergens are usually accumulated in the outer epidermal layers of plant organs, thus, patients displaying Rosaceae nsLTP-specific IgE antibodies often tolerate peeled-off fruits, and certain foods, such as carrots, potatoes, bananas, and melon. Even so, sensitized individuals may be at risk of developing severe allergenic symptoms upon ingestion of nuts.[40]

TLP or PR-5 proteins include the almond allergen Pru du 2 and other fruit proteins from the Rosaceae family such as apple (Mal d 2),[33] peach (Pru p 2)[19] or cherry (Pru av 2).[68] Additionally, this group of proteins has also been described in other fruits belonging to different botanic families, such as kiwi from Actinidiaceae family[69] and banana from Musaceae family[70]. The clinical relevance of sensitization to distinct TLP continues to be a matter of debate, though TLP found in edible fruits have been recognized as being potent food allergens, susceptible to trigger allergic reactions in sensitized individuals.[71] The presence of these proteins in almond may be responsible for some of the allergic responses associated to this seed, so further studies should be conducted to establish its relevance.

A broad spectrum of cross-reactivity between profilins of inhaled and nutritive allergenic sources has been described since homologue profilins can be virtually found in almost every plant source.[40,72,73] Considering that almond contains the panallergen Pru du 4, the risk of sensitization to multiple foods and pollens in a patient allergic to profilins is elevated.[40] Fortunately, the clinical manifestations associated to profilin allergy are considered to be mild and mainly limited to the oral cavity. Profilins are not very resistant to heat denaturation and gastric digestion, thus they cannot cause sensitization through the gastrointestinal tract, behaving as Class 2 food allergens.[61] Many profilin-sensitized patients do not exhibit symptoms.[40,44] In contrast, Asero et al.[74] demonstrated that profilins can be considered as clinically relevant food allergens in specific food-allergic patients. The overall impression from clinical studies is that patients displaying profilin-specific IgE antibodies can be either asymptomatic or at risk of developing multiple pollen-associated food allergy.

Amandin (Pru du 6) has been defined both as a major storage protein and as a major allergen in almond, being one of the first allergens to be studied in almond. Roux et al.[35] reported amandin as a major allergen related with severe reactions to almond upon ingestion. Polypeptides from amandin are highly resistant to different heat treatments during food processing[75] and the contamination of food with this allergen can lead to a significant risk of increasing the number of sensitized patients. In a recent study from Holden et al.,[76] it was suggested that amandin can possibly cross-react with α-conglutin from lupine, since this protein is another 11S globulin. In order to establish the clinical significance of this cross-reactivity, oral challenge tests in almond- and lupine-allergic patients should be performed.

The seed storage proteins 2S albumin and γ-conglutin identified in almond were characterized as IgE-binding proteins.[38] The availability of sera from allergic patients to almond, who were reactive to skin prick tests and positive-responsive to almond in oral challenge tests, permitted the isolation of these two almond allergens. However, it was emphasized that the IgE-binding and the serological reactivity of these proteins does not imply the clinical symptoms of the allergy and further studies of clinical reactivity, particularly regarding food challenges are needed.

Pru du 5 was described recently in the literature as an almond allergen. Immunoreactivity of the r60sRP was evaluated with dot blot analysis using pooled and individual sera of allergic patients, showing that the expressed Pru du 5 proteins possess the ability to bind the IgE antibodies. However, to classify it as a major allergen, further investigation is still required involving a large number of sera from almond-allergic patients.[46]

The recent research based on the characterization of allergenic components has opened new perspectives in the diagnosis of food allergy. The possibility of using a large number of single allergenic proteins, either *in vivo* or *in vitr*o, diagnosing food allergy at a molecular level, will have a considerable impact on the clinical management of food allergies in the near future. More collaborative studies between clinicians and researchers should be encouraged, since those would certainly enable better knowledge about the mechanisms of reaction of each specific group of allergens, their clinical manifestations and the best preventive treatments for allergic patients.

DETECTION OF ALMOND AND OTHER FOOD ALLERGENS

The need for adequate methodology to detect food allergens has been rapidly increasing over the last years, especially in response to the demands imposed by the current legislation. Food industry has been addressing with special interest the necessities of food allergic consumers, not only concerning the proper food labeling, but also minimizing allergen cross-contamination among foodstuffs. Therefore, suitable analytical methods are required to detect allergenic proteins, as they are mostly present at trace levels.[77] The requirements needed for detecting allergenic ingredients in food involve appropriate specificity and sensitivity to trace minute amounts of the target allergens or the correspondent markers in complex food matrices, including processed foods.

The determination of upper limits for allergenic non-ingredient food components would be an important progress for the protection of allergic consumers. Nevertheless, these limits are only meaningful with the development of adequate analytical methodologies to verify their compliance.[78] According to Poms et al. [79] the ideal limit of detection (LOD) for allergens in food products should range between 1 and 100 mg/kg. The 'food allergy' working group of the German Society for Allergology and Clinical Immunology and the Association of German Allergologists proposed upper limits of 10-100 mg/kg of the allergenic food or 1-10 mg/kg of the protein fraction of the allergenic food, depending on its allergenicity, that would protect most allergic consumers from severe allergic reactions.[78] The study performed by Morrisset et al.[80] to establish the thresholds of clinical reactivity to milk, egg, peanut and sesame in allergic patients suggested that detection tests should ensure a sensitivity of 10 mg/kg, 24 mg/kg and 30 mg/kg for egg, peanut and milk proteins, respectively, in order to guaranty a 95% safety for patients who are allergic to the referred foods, and on the basis of consumption of 100 g of food. In the specific case of oil allergies, the limit of sensitivity should fall to 5 mg/kg.

One major prerequisite for the development of analytical methods, including allergen detection techniques, is the availability of certified reference materials (CRM). The existing materials from the Institute for Reference Materials and Measurements (IRMM, Geel) for peanut testing (IRMM-481) consist of five distinct types of peanut powders with different varieties and geographical origin, but are not reference or certified materials. Concerning tree nuts, there are no test materials yet available supplied by the IRMM. Presently, an accredited Greek laboratory[81] has released a set of testing reference materials for the detection of some tree nut allergens such as almond, hazelnut and walnut, but lacking appropriate stability assays.

Several methods for almond detection have been developed, mainly relying on immunochemical and DNA-based techniques. The immunochemical methods include enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD), dipsticks tests, immunoblotting and biosensors. These methods have been successfully applied for the detection of allergens in food with the specificity based on the precise binding between epitopes present on the target protein and an immunoglobulin. Nevertheless, the use of immunoassays has numerous problems mainly due to the cross-reactivity with non-target proteins and to the low resistance of proteins to food processing, since it can cause conformational changes in the tri-dimensional structure of the epitopes (e.g. heat induced denaturation) and/or protein cleavage, affecting linear epitopes (e.g. fermentation).[82] During the thermal processing of foods, several interactions between food allergens and other molecular components can occur, such as protein modifications induced by Maillard reactions. Until now, little is known about how thermal processing, Maillard reactions and other possible chemical modifications can influence the performance of commercially available immunoassay kits for the detection of allergens in foods.[83,84] In addition, it is important to refer that the solubility of a protein is also affected by chemical modifications (progressive Maillard reactions), conditioning the extractability of this analyte from the food matrix. All these factors may, consequently, contribute to the low reproducibility, as well as increased chances of false negative results observed with immunoassays (ELISA kits) since they are based on a analyte-receptor binding.[84] Other interesting point about the immunoassays lies on the type of allergens used to produce the antibodies. Some authors suggest that the antibodies used in ELISA kits are produced in different conditions, and the accuracy of these methods can be affected by them.[84]

Lately, the DNA-based methods have been increasingly used as highly sensitive and specific alternatives for allergen detection, taking advantage of the greater thermal stability of DNA molecules compared to proteins. These techniques rely on the use of polymerase chain reaction (PCR), either as qualitative end-point PCR or quantitative real-time PCR assays. The specificity is achieved using primers and probes specifically designed for the gene encoding the allergen or marker protein. Methods combining both PCR and ELISA have been developed for the detection of food allergens to fit the labeling requirements imposed by the legislation.[85]

Immunochemical methods. ELISA is probably the immunoassay most widely used for the detection of food allergens. It relies on the specific interaction between the antibody and the antigen, which is the allergen or marker protein in the case of food allergen detection. There are different types of ELISA tests (sandwich, competitive and indirect) available for food analysis, but the most commonly chosen is the sandwich-ELISA. Immunoassays can provide qualitative or quantitative results. In qualitative tests, the results are expressed simply as positive or negative, whereas in quantitative ELISA, the optical or fluorescent signals of the unknown samples are compared with standard curves consisting of known quantities of target proteins serially diluted. **Table 2** presents a set of commercially available ELISA kits for the detection of almond allergens. These tests present the advantages of rapid performance and versatility, being extensively applied with reliable results and LOD down to 0.1 mg/kg of almond protein in food samples within 30-35 minutes.[86-88] In almond, almost 95% of the total protein content is water soluble, making them easily accessible, which contributes to the frequent use of ELISA tests for almond protein detection.[88]

Roux et al.[51] reported the development of a competitive ELISA for the detection of the major allergen of almond, amandin or almond major protein (AMP), since this protein accounts for approximately 65-75% of total almond protein, presenting high thermal stability.

Table 2. Commercial immunoassays for the detection of almond allergens

Commercial kits	Assay type	Brand	LOD (mg/kg)	Time for sample testing (min)	Catalogue number
Rapid-3-D Almond Test Kit	Lateral flow device (Positive/Negative)	Tepnel	1	10	902086G
Reveal 3-D Almond Test	Lateral flow device (Positive/Negative)	NEOGEN corporation	5	10	902086G
BioKits Almond Assay Kit	Polyclonal antibodies to almond protein, non-competitive sandwich type ELISA	NEOGEN corporation	0.1	90	902083N
BioKits Almond Assay Kit	Polyclonal antibodies to almond protein, non-competitive sandwich type ELISA	Tepnel	0.1	90	902083N
Alert Almond Assay Kit	Sandwich ELISA	NEOGEN corporation	5	30	8441
RIDASCREEN FAST Mandel/Almond	Polyclonal antibody specifically for almond protein detection, sandwich ELISA	R-Biopharm AG	1.7	30 (sample extraction)	R6901
ELISA Systems Almond	ELISA	ELISA Systems		35 (sample extraction)	95200 ESARD-48
Veratox for Almond Allergen	Sandwich ELISA	NEOGEN corporation	2.5	30	8440

The proposed method was considered very sensitive (detection of 5-37 mg/kg of AMP in several spiked foods) and specific, presenting only minor cross-reactivity with some globulins and albumins from other nuts and legumes.[51] Rejeb et al.[89] developed a multiresidue methodology based on competitive indirect ELISA, which allowed the simultaneous determination of almond, peanut, hazelnut, Brazil nut and cashew nut with a LOD of 1 mg/kg of target protein in chocolate samples. Garber et al.[87] compared three commercial sandwich-ELISA test kits for the detection of hazelnuts and almonds. The determined LOD and dynamic ranges for almonds spiked into cooked oatmeal, dipping chocolate and muffins (baked) varied from 3 to 39 mg/kg, depending on the food matrix and on the tested ELISA kit.[87]

Lateral flow devices (LFD) or dipstick assays are another type of immunochemical tests applied to the detection of allergens in food. They are based on the same principle as ELISA, but with simpler and faster performance (~10 minutes) making them quite often used by the industry for rapid food screening.[88] The results are mainly qualitative or semi-quantitative and can be interpreted visually. Like the ELISA tests, there are two types of LFD, the sandwich and the competitive formats. Associated to this type of assays, some drawbacks can be pointed out due to the susceptibility of these devices in providing false negative results as well as the lack of quantitative information.[90] Recently, several commercial kits have become available for the quick on-site detection of food allergens, including the LFD tests that provide rapid information about the presence of certain allergens within a few minutes. The application of LFD to foods can allow the detection of almond protein down to 1 mg/kg in less than 10 minutes.[88] **Table 2** lists the commercially available LFD and the ELISA kits for the detection of almond allergens in raw and processed foodstuffs.

Another protein-based method for food allergen detection consists of the use of immunoblotting as a very reliable tool, although not adequate for routine analysis. It constitutes a choice for confirmatory testing of the presence of allergens in food, allowing the characterization of IgE from sensitized individuals and the evaluation of antibody specificity. Scheibe et al.[91] have described a sensitive protocol for the detection of almond in chocolates using SDS-immunoblot with a chemiluminescence detection method with a LOD of 5 mg/kg of almond protein in chocolate.

Biosensors, for their characteristics of fast response time and low cost, are very attractive platforms for new applications in different emerging fields such as allergen detection. They are analytical devices consisting of a biological recognition element (e.g. cells, proteins and oligonucleotides) in direct contact with a transducer that produces the signal. Immunochemical sensors are able to measure interactions between different molecules in real-time and can be applied for the detection and quantitation of food allergens.[92] The antibody-allergen interaction can be detected by different types of transducers (optical, acoustical, amperometrical or potentiometrical), producing a signal that is further processed to give a proportional output to the concentration of a specific analyte. The optical biosensors base their function on the phenomenon of surface plasmon resonance (SPR). Their application was demonstrated for peanut detection[93,94] and other allergens from milk, egg, hazelnut, shellfish and sesame, reaching levels of detection comparable to the most sensitive ELISA.[94] More recently, Bremer et al.[95] developed a rapid and sensitive direct biosensor immunoassay based on a highly specific monoclonal antibody to identify the presence of hazelnut proteins in olive oils. Biosensors have several potential applications, although only few have been proposed for food allergen detection and, to our knowledge, none of them targeting almond allergens. More studies are still required to fully understand its potential utilization for monitoring the presence of allergens in food, namely tree nut detection.

Mass-spectrometry based methods. One of the current problems associated with the detection of allergenic proteins and peptides relies on their identification. In this regard, mass spectrometry-based methodologies have demonstrated their usefulness in obtaining information for the identification of allergenic proteins.[96] Mass spectrometry (MS) methods can overcome the drawbacks of cross-reactivity phenomena of immunoassays and the inability of DNA techniques to directly detect the allergenic protein. Advantages of MS rely on the unambiguous confirmation by proteins/peptides. Information about molecular mass is provided and protein identification can be carried out by means of database search algorithms using the number of matching sequences, fragments and peptides.[96] The identification of proteins by MS technology is usually performed using the “bottom up” approach that is conducted based on the digestion of proteins with a specific protease, commonly trypsin. Mass spectra are recorded after the separation of proteolytic fragments by reversed-phase HPLC.[97] Considering the diversity of allergenic molecules, the process of purification is specifically developed to guaranty unambiguous recognition of the molecule by the generation of a peptide mass fingerprinting. Additionally, in the case of processed foods whose pattern of proteins/peptides might be altered, MS approach often provides insights on the nature of protein modifications readily elucidated by MS and MS/MS spectra.[96]

Some applications using liquid chromatography coupled to MS have been reported to detect hidden food allergens mainly from peanut.[98-101] Only very recently, the detection of food allergens from tree nuts was reported. Bignardi et al.[102] successfully applied a method based on liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) for the simultaneous detection of five allergens (Ana o 2, Cor a 9, Pru 1, Jug r 4 and Ara h3/4) from cashew, hazelnut, almond, walnut and peanut, respectively, in food matrices. The assays allowed the detection and quantitation of Pru 1 protein down to the levels of 17 mg/kg and 58 mg/kg, respectively, in biscuits. Another approach including the detection of tree nut allergens consisted of a multi-method to detect seven allergens based on liquid chromatography and triple-quadrupole tandem MS.[103] The use of marker peptides implemented in multiple detection mode was capable of simultaneously identifying milk, egg, soy, hazelnut, peanut, walnut and almond in concentrations ranging from 10 to 1000 mg/kg in incurred bread material. Regarding almond detection, four different marker peptides were used to target prunin as the target allergen, from which one enabled a LOD of 3 mg/kg of almond in bread material.

DNA-based methods. These techniques consist of the specific amplification of a gene fragment encoding a protein from the allergenic ingredient by means of PCR, whose specificity is achieved by the use of primers and, frequently, probes. Although these methods do not target directly the offending proteins, they are considered to be very sensitive and specific, taking advantage of the elevated stability of DNA molecules at high temperatures and their resistance to high pH values. In addition, the DNA-based methods can be included in routine analysis and act as a confirmatory tool, when adequate immunoassays do not exist.

In spite of the advantages of DNA-based methods, PCR is still much contested because when detecting a gene encoding for an allergen, it does not necessarily imply its expression. Consequently, the results obtained by DNA detection do not account for the actual allergenic potential. However, the same happens with some, if not most, ELISA tests that do not necessarily detect the allergenic proteins, but rather species-specific protein markers. In fact, the detection of a molecular marker gives indirect information of the allergenic potential, but provides the presence of the allergenic ingredient.[104]

Recent reviews have demonstrated the increased number of applications of DNA-based methods and their suitability to detect food allergens.[82,96,105] In these methods, the specific target is amplified either by end-point PCR, being distinguished on the basis of their differential migration through agarose gel electrophoresis, or by real-time PCR using fluorescent labeled probes or dyes. Other PCR-based approaches such as ligation-dependent probe amplification (LPA)[106] and the combination of PCR amplification with ELISA have also been successfully implemented to detect food allergens.[85] Nevertheless, real-time PCR has been so far the most widely applied PCR strategy to detect food allergens. Several real-time PCR approaches have been proposed to detect food allergens from peanuts,[107,108-112] celery,[107,113] mustard,[113] lupine,[114,115] sesame,[109,113,116] and tree nuts including hazelnut,[107,104,109,116-119] walnut,[109,120] macadamia,[121] pecan,[122] pistachio,[123] cashew nut,[109,124] Brazil nut[125,126] and almonds.[107,109,127-129]

Pafundo et al.[127] developed two systems for the detection of almond allergens using SYBR GreenER real-time PCR. The systems specifically targeted the genes encoding for the allergenic protein Pru 1 (prunin), that is the major component of amandin, and the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) of *Prunus persica*, as marker for *Prunus* detection, since it is a multi-copy chloroplastic sequence. The development of the two systems allowed the detection of Pru-1 in biscuits containing processed almond down to 1 DNA copy. In another work, the same authors reported the development of a multiple-target assay based on SYBR GreenER real-time multiplex PCR to detect sesame, peanut, cashew nut, hazelnut, walnut and almond.[109] This method enabled the detection of low quantities of almond DNA (5 pg), with LOD ranging from 1 to 100 mg/kg of almond in spiked biscuits.[109] Köppel et al.[107] presented two tetraplex real-time PCR systems for the detection of eight allergens in food based on the application of TaqMan probes. The proposed systems were called “AllAll A” and “AllAll B”. “AllAll A” allowed the simultaneous detection of DNA from peanut, hazelnut, celery and soy, while “AllAll B” enabled the detection of milk, sesame, egg and almond in food. The assays exhibited good specificity and sensitivity in the range of 0.01% of target ingredient in rice cookies. Concerning the specific detection of almond, a LOD of 10 mg/kg was obtained for almond spiked in rice cookies. In the same work, the PCR results, when compared to ELISA, seemed to indicate a correlation between both methods, though more investigation is needed to support this suggestion.[107] Röder et al.[128] have also developed a method based on real-time PCR system with TaqMan probes to detect almond allergen nsLTP (Pru du 3), down to a LOD of 5 mg/kg of almond in a variety of food matrices. In this study, the PCR results were matched with those of ELISA within the known limits of variation for these tests in spiked levels over 100 mg/kg, allowing establishing a qualitative correlation between the developed real-time PCR system and two commercial ELISA kits.[128] Another study regarding the detection of a different almond allergen (Pru du 5) was proposed by Costa et al.[129] by means of high resolution melting (HRM) analysis in a real-time PCR system with Evagreen DNA binding dye. The authors reported the detection of the gene encoding for Pru du 5 allergen with a relative LOD of 50 mg/kg of almond in walnut material and an absolute LOD of 10 pg of almond DNA. The application of HRM analysis for almond detection allowed distinguishing almond from other fruits from the Rosaceae family such as peach, apricot and nectarine.[129]

An important issue concerns the effect of matrix on allergen detection. A comparative evaluation of ELISA and real-time PCR techniques in detecting and correctly quantitating hazelnut in food model systems was recently described by Platteau et al.[117] The authors demonstrated that food processing has an impact on hazelnut detection in cookies and cookie ingredients using real-time PCR as well as ELISA. They further indicated that both methods lacked robustness with regard to food processing, without drawing any firm conclusion about the most suited technique to detect hazelnut in processed foods, highlighting the need for adequate reference materials.

SUMMARY

In the recent years, some studies have been performed in order to characterize the allergenic proteins present in almond. To our knowledge, currently, eight groups of allergens have been identified and characterized, as well as the respective allergenic isoforms. Although some of them have not yet been well defined concerning their clinical implications in sensitized individuals, most are known to trigger severe adverse reactions and are susceptible for cross-reactivity with homologous allergens among other fruits from the Rosaceae family. Furthermore, an adequate characterization of the allergenic components of almond could provide new insights in the diagnosis of almond allergy and facilitate the development of preventive treatments.

Almonds are frequently subjected to harsh processing conditions prior to or during their incorporation into foods. Protein denaturation, aggregation, and structure disruption can be promoted by thermal/chemical treatments, having a potential to modify allergenic properties of almond proteins. In this context, molecular characterization studies on almond allergens are also important issues since functionality and immunoreactivity of a protein is closely linked to its conformation. Thus, structural changes induced by thermal and/or chemical denaturation should be studied to provide important information regarding its global stability, which may help explaining changes in allergenicity that occur as a result of food processing.

Food allergy with respect to almond is an important health problem due to its wide use in the food industry and, consequently, considered as a potential source of hidden allergens derived from the incorrect labeling or unintentionally inclusion via improper clean-up and cross-contamination in the processing system. On the other hand, to comply with legislation, excessive labeling about the presence of potential allergens in foodstuffs may also contribute to restrict the range of adequate foods for allergic individuals.

As a consequence of the established clear guidelines on food allergen labeling, an increasing need for the development of suitable analytical methods has arisen. The immunoassays, such as ELISA for the detection of food allergens, are probably the most widely used techniques due to their high sensitivity and specificity to target the offending proteins. To overcome the problems associated with the immunochemical assays, namely cross-reactivity, reduced protein solubility and degradation caused by food processing, the DNA-based methods have emerged as proper alternatives to detect food allergens without the need for adequate antibodies. However, recent reports have demonstrated that DNA analysis is also affected by food matrix and processing leading to incorrect quantitation. In our opinion the effect of food processing on the recovery and actual levels of detection for both DNA and protein methods should be adequately addressed in more future research. Another important issue, in the case of DNA methods, concerns the choice of adequate extraction protocols to obtain DNA extracts from complex food matrices, free of PCR inhibitors, maximizing the assay sensitivity.

Regarding the major requirement for allergen identification, MS-based methods through the combination of liquid chromatography with MS-detection have emerged as reliable tools for unambiguous identification of proteins or peptides from allergenic foods with potential for quantitative analysis and for detecting changes after food processing. MS methods overcome the biggest problems of ELISA (cross-reactivity issues) and PCR (indirect identification of the target allergen), allowing direct detection of proteins without the need for antibodies and with potential for the simultaneous analysis of multiple allergens.

Several protein and DNA-based methodologies have become available for the detection of allergenic ingredients in food, but the question about the most appropriate technique for allergen detection and quantitation is yet a matter of debate. Opinions continue to diverge about the best target analyte (protein or DNA molecules) to be used and on the best methods to detect them on a routine analysis basis. Official guidelines should be implemented shortly, regulating limits for the presence of potentially allergenic ingredients in pre-packaged food and the recommended methodology for its monitoring.

Considering that the only effective method to manage food allergies for sensitized consumers at the present is the avoidance of foods containing the provocative proteins, analytical methodologies to detect food allergens at trace levels have gained utmost importance. They range from well-documented protocols to newly developed tools, but reference methods, which are always needed to standardize procedures in the development of other analytical assays are still lacking. To support this requirement, the rapid development of reference materials is of high priority.

Finally, clinicians and food chemists should become closer to work on harmonization of procedures that can provide better understanding in clinical allergy tests and food analysis.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidized by FSE and MCTES).

REFERENCES

(1) INE, Instituto Nacional de Estatística; http://www.ine.pt (Accessed: 19th January 2012).

(2) FAO, Food and Agriculture Organization ProdStat Database; http://faostat.fao.org (Accessed: 19th January 2012).

(3) Alasalvar, C.; Shahidi, F. Tree Nuts: Composition, Phytochemicals, and Health Effects: an overview. In *Tree Nuts: Composition, Phytochemicals, and Health Effects*; Alasalvar, C., Shahidi, F., Eds.; CRC Press: Boca Raton, FL, 2008; pp 1-6.

(4) FDA, Administration Qualified Health Claims: Letter of Enforcement Discretion-Nuts and Coronary Heart Disease, Docket No 02P-0505, Food and Drug Administration, Washington DC, 2003.

(5) Sathe, S.; Sharma, G.; Roux, K. Tree Nut Allergens. In *Tree Nuts: Composition, Phytochemicals, and Health Effects*; Alasalvar, C., Shahidi, F., Eds.; CRC Press: Boca Raton, FL, 2008; pp 65-83.

(6) Sicherer, S. H.; Muñoz-Furlong, A.; Godbold, J. H.; Sampson, H. A. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol*. **2010**, *125*, 1322-1326.

(7) Sicherer, S. H.; Muñoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: A 5-year follow-up study. *J. Allergy Clin. Immunol*. **2003**, *112*, 1203-1207.

(8) Burney, P.; Summers, C.; Chinn, S.; Hooper, R.; van Ree, R.; Lidholm, J. Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy*, **2010**, *65*, 1182-1188.

(9) CODEX STAN 1. Amended in 1991, 1999, 2001, 2003, 2005, 2008 and 2010 regarding the general standard for the labelling of prepackaged foods. FAO/WHO Standards. *Off. Codex Stand.*, **1985**.

(10) Directive 2000/13/EC relating to the labelling, presentation and advertising of foodstuffs. *Off. J. Eur. Union* **2000**, *L109*, 29-42.

(11) Directive 2003/89/EC amending Directive 2000/13/EC regarding indication of the ingredients present in foodstuffs. *Off. J. Eur. Union* **2003**, *L308*, 15-18.

(12) Commission Directive 2007/68/EC amending Annex IIIa to Directive 2000/13/EC regarding certain food ingredients. *Off. J. Eur. Union* **2007**, *L310*, 11-14.

(13) Sicherer, S. H.; Sampson, H. A. Food allergy. *J. Allergy Clin. Immunol*. **2006**, *117*, S470-S475.

(14) Sampson, H. A. Anaphylaxis and Emergency Treatment. *Pediatrics* **2003**, *111*, 1601-1608.

(15) Sicherer, S. H. Epidemiology of food allergy. *J. Allergy Clin. Immunol*. **2011**, *127*, 594-602.

(16) Bock, S. A.; Muñoz-Furlong, A.; Sampson, H. A. Further fatalities caused by anaphylactic reactions to food, 2001-2006. *J. Allergy Clin. Immunol*. **2007**, *119*, 1016-1018.

(17) Pumphrey, R. S. H.; Gowland, M. H. Further fatal allergic reactions to food in the United Kingdom, 1999-2006. *J. Allergy Clin. Immunol*. **2007**, *119*, 1018-1019.

(18) Fernández-Rivas, M.; Ballmer-Weber, B. Food allergy. In *Managing Allergens in Food*; Mills, C., Wichers, H., Hoffmann-Sommergruber, K., Eds.; Woodhead Publishing Group and CRC Press: Boca Raton, 2007; pp 3-28.

(19) Chen, L.; Zhang, S.; Illa, E.; Song, L.; Wu, S.; Howad, W.; Arus, P.; Weg, E.; Chen, K.; Gao, Z. Genomic characterization of putative allergen genes in peach/almond and their synteny with apple. *BMC Genomics* **2008**, *9*, 543.

(20) Shahidi, F.; Zhong, Y.; Wijeratne, S.; Ho, C.-T. Almond and Almond Products: Nutraceutical components and health effects. In *Tree Nuts: Composition, Phytochemicals, and Health Effects*; Alasalvar, C., Shahidi, F., Eds.; CRC Press: Boca Raton, FL, 2008; pp 127-141.

(21) Rodrigues, J.; Crespo, J. F.; Lopez-Rubio, A.; de la Cruz-Bertolo, J.; Ferrando-Vivas, P.; Vives, R.; Daroca, P. Clinical cross-reactivity among foods of the Rosaceae family. *J. Allergy Clin. Immunol.* **2000**, *106*, 183-189.

(22) Sicherer, S. H.; Furlong, T. J.; Muñoz-Furlong, A.; Burks, A. W.; Sampson, H. A. A voluntary registry for peanut and tree nut allergy: Characteristics of the first 5149 registrants. *J. Allergy Clin. Immunol.* **2001**, *108*, 128-132.

(23) Tawde, P.; Venkatesh, Y. P.; Wang, F.; Teuber, S. S.; Sathe, S. K.; Roux, K. H. Cloning and characterization of profilin (Pru du 4), a cross-reactive almond (*Prunus dulcis*) allergen. *J. Allergy Clin. Immunol.* **2006**, *118*, 915-922.

(24) Allergen Nomenclature, Allergen Nomenclature IUIS Allergen Nomenclature Sub-Committee; http://www.allergen.org (Accessed: 19th January 2012).

(25) NCBI, National Center for Biotechnology Information. Bethesda, Maryland, USA; http://www.ncbi.nlm.nih.gov (Accessed: 19th January 2012).

(26) Breiteneder, H. Classifying food allergens. In *Detecting allergens in food*; Koppelman, S. J., Hefle, S. L., Eds.; CRC Press: Boca Raton, FL, 2006.

(27) Markovic-Housley, Z.; Degano, M.; Lamba, D.; von Roepenack-Lahaye, E.; Clemens, S.; Susani, M.; Ferreira, F.; Scheiner, O.; Breiteneder, H. Crystal Structure of a Hypoallergenic Isoform of the Major Birch Pollen Allergen Bet v 1 and its Likely Biological Function as a Plant Steroid Carrier. *J. Mol. Biol.* **2003**, *325*, 123-133.

(28) Mills, C. E.; Sancho, A. I.; Moreno, J.; Kostyra, H. The effects of food processing on allergens. In *Managing Allergens in Food*; Mills, C., Wichers, H., Hoffmann-Sommergruber, K., Eds.; CRC Press: Boca Raton, FL, 2007; pp 117-133.

(29) Vanek-Krebitz, M.; Hoffmann-Sommergruber, K.; Laimer da Camara Machado, M.; Susani, M.; Ebner, C.; Kraft, D.; Scheiner, O.; Breiteneder, H. Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochem. Biophys. Res. Commun.* **1995**, *214*, 538-551.

(30) Karamloo, F.; Scheurer, S.; Wangorsch, A.; May, S.; Haustein, D.; Vieths, S. Pyr c 1, the major allergen from pear (*Pyrus communis*), is a new member of the Bet v 1 allergen family. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *756*, 281-293.

(31) Scheurer, S.; Metzner, K.; Haustein, D.; Vieths, S. Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. *Mol. Immunol.* **1997**, *34*, 619-629.

(32) Vieths, S.; Scheurer, S.; Ballmer-Weber, B. Current understanding of cross-reactivity of food allergens and pollen. *Ann. N. Y. Acad. Sci.* **2002**, *964*, 47-68.

(33) Breiteneder, H. Thaumatin-like proteins - a new family of pollen and fruit allergens. *Allergy* **2004**, *59*, 479-481.

(34) Shewry, P. R.; Halford, N. G. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* **2002**, *53*, 947-958.

(35) Roux, K. H.; Teuber, S. S.; Sathe, S. K. Tree Nut Allergens. *Int. Arch. Allergy Immunol.* **2003**, *131*, 234-244.

(36) Moreno, F. J.; Clemente, A. 2S Albumin Storage Proteins: What makes them food allergens? *Open Biochem. J.* **2008**, *2*, 16-28.

(37) Aalberse, R. C. Structural biology of allergens. *J. Allergy Clin. Immunol.* **2000**, *106*, 228-238.

(38) Poltronieri, P.; Cappello, M.; Dohmae, N.; Conti, A.; Fortunato, D.; Pastorello, E. A.; Ortolani, C.; Zacheo, G. Identification and characterisation of the IgE-binding proteins 2S albumin and conglutin gamma in almond (*Prunus dulcis*) seeds. *Int. Arch. Allergy Immunol.* **2002**, *128*, 97-104.

(39) Clemente, A.; Chambers, S. J.; Lodi, F.; Nicoletti, C.; Brett, G. M. Use of the indirect competitive ELISA for the detection of Brazil nut in food products. *Food Control* **2004**, *15*, 65-69.

(40) Hauser, M.; Roulias, A.; Ferreira, F.; Egger, M. Panallergens and their impact on the allergic patient. *Allergy Asthma Clin. Immunol.* **2010**, *6*, 1-14.

(41) Staiger, C. J.; Goodbody, K. C.; Hussey, P. J.; Valenta, R.; Drøbak, B. K.; Lloyd, C. W. The profilin multigene family of maize: differential expression of three isoforms. *Plant J.* **1993**, *4*, 631-641.

(42) Westphal, S.; Kempf, W.; Foetisch, K.; Retzek, M.; Vieths, S.; Scheurer, S. Tomato profilin Lyc e 1: IgE cross-reactivity and allergenic potency. *Allergy* **2004**, *59*, 526-532.

(43) Hauser, M.; Egger, M.; Wallner, M.; Wopfner, N.; Schmidt, G.; Ferreira, F. Molecular properties of plant food allergens: a current classification into protein families. *Open Immunol. J.* **2008**, *1*, 1-12.

(44) Wensing, M.; Akkerdaas, J. H.; van Leeuwen, W. A.; Stapel, S. O.; Bruijnzeel-Koomen, C. A. F. M.; Aalberse, R. C.; Bast, B. J. E. G.; Knulst, A. C.; van Ree, R. IgE to Bet v 1 and profilin: Cross-reactivity patterns and clinical relevance. *J. Allergy Clin. Immunol.* **2002**, *110*, 435-442.

(45) Mills, C. E.; Jenkins, J.; Robertson, J.; Griffiths-Jones, S.; Shewry, P. Identifying allergenic proteins in food. In *Pesticide, veterinary and other residues in food*; Watson, D. H., Ed.; CRC Press: Boca Raton, FL, 2004.

(46) Abolhassani, M.; Roux, K. H. cDNA Cloning, expression and characterization of an allergenic 60s ribosomal protein of almond (*Prunus dulcis*). *Iran. J. Allergy Asthma Immunol.* **2009**, *8*, 77-84.

(47) López-Matas, M. A.; Ferrer, A.; Larramendi, C. H.; Huertas, A. J.; Pagán, J. A.; García-Abujeta, J. L.; Bartra, J.; Andreu, C.; Lavín, J. R.; Carnés, J. Acidic ribosomal protein 60S: A new tomato allergen. *Food Chem.* **2011**, *127*, 638-640.

(48) Mills, E. N.; Jenkins, J.; Marigheto, N.; Belton, P. S.; Gunning, A. P.; Morris, V. J. Allergens of the cupin superfamily. *Biochem. Soc. Trans.* **2002**, *30*, 925-929.

(49) Garcia-Mas, J.; Messeguer, R.; Arús, P.; Puigdomènech, P. Molecular characterization of cDNAs corresponding to genes expressed during almond (*Prunus amygdalus* Batsch) seed development. *Plant Mol. Biol.* **1995**, *27*, 205-210.

(50) Albillos, S. M.; Jin, T.; Howard, A.; Zhang, Y.; Kothary, M. H.; Fu, T.-J. Purification, crystallization and preliminary X-ray characterization of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. *J. Agric. Food Chem.* **2008**, *56*, 5352–5358.

(51) Roux, K. H.; Teuber, S. S.; Robotham, J. M.; Sathe, S. K. Detection and stability of the major almond allergen in foods. *J. Agric. Food Chem.* **2001**, *49*, 2131-2136.

(52) Sathe, S. K.; Wolf, W. J.; Roux, K. H.; Teuber, S. S.; Venkatachalam, M.; Sze-Tao, K. W. C. Biochemical Characterization of amandin, the major storage protein in almond (*Prunus dulcis* L.). *J. Agric. Food Chem.* **2002**, *50*, 4333-4341.

(53) Jin, T.; Albillos, S. M.; Guo, F.; Howard, A.; Fu, T.-J.; Kothary, M. H.; Zhang, Y.-Z. Crystal structure of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. *J. Agric. Food Chem.* **2009**, *57*, 8643-8651.

(54) Gekko, K.; Timasheff, S. N. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* **1981**, *20*, 4667-4676.

(55) Albillos, S. M.; Menhart, N.; Fu, T.-J. Structural Stability of Amandin, a Major Allergen from Almond (*Prunus dulcis*), and Its Acidic and Basic Polypeptides. *J. Agric. Food Chem.* **2009**, *57*, 4698-4705.

(56) Shewry, P. R.; Napier, J. A.; Tatham, A. S. Seed Storage Proteins: Structures and Biosynthesis. *Plant Cell* **1995**, *7*, 945-956.

(57) Burks, A. W.; Williams, L. W.; Helm, R. M.; Connaughton, C.; Cockrell, G.; O'Brien, T. (). Identification of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J. Allergy Clin. Immunol.* **1991**, *88*; 172-179.

(58) Burks, A. W.; Brooks, J. R.; Sampson, H. A. Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J. Allergy Clin. Immunol.* **1988**, *81***,** 1135-1142.

(59) Wang, F.; Robotham, J. M.; Teuber, S. S.; Tawde, P.; Sathe, S. K.; Roux, K. H. Ana o 1, a cashew (*Anacardium occidental*) allergen of the vicilin seed storage protein family. *J. Allergy Clin. Immunol.* **2002**, *110*, 160-166.

(60) Kolivas, S.; Gayler, K. R. Structure of the cDNA coding for conglutin gamma, a sulphur-rich protein from *Lupinus angustifolius*. *Plant Mol. Biol.* **1993**, *21*, 397-401.

(61) Breiteneder, H.; Ebner, C. Molecular and biochemical classification of plant-derived food allergens. *J. Allergy Clin. Immunol.* **2000**, *106*, 27-36.

(62) Bolhaar, S. T.; van Ree, R.; Ma, Y.; Bruijnzeel-Koomen, C. A.; Vieths, S.; Hoffmann-Sommergruber, K.; Knulst, A. C.; Zuidmeer, L. Severe allergy to sharon fruit caused by birch pollen. *Int. Arch. Allergy Immunol.* **2005**, *136*, 45-52.

(63) Kosma, P.; Sjölander, S.; Landgren, E.; Borres, M. P.; Hedlin, G. Severe reactions after intake of soy drink in birch pollen allergic children sensitized to Gly m 4. *Acta Paediatr*. **2011**, *100*, 305-307.

(64) Andersen, M. B.; Hall, S.; Dragsted, L. O. Identification of European allergy patterns to the allergen families PR-10, LTP, and profilin from Rosaceae fruits. *Clin. Rev. Allergy Immunol.* **2011**, *41*, 4-19.

(65) Fernández-Rivas, M. Reactividad cruzada en frutas y vegetales. *Allergol. Immunopathol.* (Madr) **2003**, *31*, 141-146.

(66) Fernández-Rivas, M.; van Ree, R.; Cuevas, M. Allergy to Rosaceae fruits without related pollinosis. *J. Allergy Clin. Immunol.* **1997**, *100*, 728-733.

(67) Pastorello, E. A.; Pravettoni, V.; Trambaioli, C.; Pompei, C.; Brenna, O.; Farioli, L.; Conti, A. Lipid transfer proteins and 2S albumins as allergens. *Allergy* **2001**, *56*, 45-47.

(68) Dall'Antonia, Y.; Pavkov, T.; Fuchs, H.; Breiteneder, H.; Keller, W. Crystallization and preliminary structure determination of the plant food allergen Pru av 2. *Acta Crystallogr. F-Struct. Biol. Cryst. Commun.* **2005**, *61*, 186-188.

(69) Gavrović-Jankulović, M.; Ćirković, T.; Vucković, O.; Atanasković-Marković, M.; Petersen, A.; Gojgić, G.; Burazer, L.; Jankov, R. M. Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J. Allergy Clin. Immunol.* **2002**, *110*, 805-810.

(70) Menu-Bouaouiche, L.; Vriet, C.; Peumans, W. J.; Barre, A.; Van Damme, E. J. M.; Rougé, P. A molecular basis for the endo-beta 1,3-glucanase activity of the thaumatin-like proteins from edible fruits. *Biochimie.* **2003**, *85*, 123-131.

(71) Hoffmann-Sommergruber, K. Plant allergens and pathogenesis-related proteins. *Int. Arch. Allergy Immunol.* **2000**, *122*, 155-166.

(72) Asero, R. Plant Food Allergies: a suggested approach to allergen-resolved diagnosis in the clinical practise by identifying easily available sensitization markers. *Int. Arch. Allergy Immunol.* **2005**, *138*, 1-11.

(73) Bonds, R. S.; Midoro-Horiuti, T.; Goldblum, R. A structural basis for food allergy: the role of cross-reactivity. *Curr. Opin. Allergy Clin. Immunol.* **2008**, *8*, 82-86.

(74) Asero, R.; Monsalve, R.; Barber, D. Profilin sensitization detected in the office by skin prick test: a study of prevalence and clinical relevance of profilin as a plant food allergen. *Clin. Exp. Allergy* **2008**, *38*, 1033–1037.

(75) Venkatachalam, M.; Teuber, S. S.; Roux, K. H.; Sathe, S. K. Effects of roasting, blanching, autoclaving, and microwave heating on antigenicity of almond (*Prunus dulcis L.*) proteins. *J. Agric. Food Chem.* **2002**, *50*, 3544-3548.

(76) Holden, L.; Sletten, G. B.; Lindvik, H.; Faeste, C. K.; Dooper, M. M. Characterization of IgE binding to lupin, peanut and almond with sera from lupin-allergic patients. *Int. Arch. Allergy Immunol.* **2008**, *146*, 267-276.

(77) Baumgartner, S.; Krska, R.; Welzig, E. Detecting allergens in foods. In *Managing Allergens in Food*; Mills, C., Wichers, H., Hoffmann-Sommergruber, K., Eds.; CRC Press: Boca Raton, FL, 2007; pp 228-250.

(78) Crevel, R. W.; Ballmer-Weber, B. K.; Holzhauser, T.; Hourihane, J. O.; Knulst, A. C.; Mackie, A. R.; Timmermans, F.; Taylor, S. L. Thresholds for food allergens and their value to different stakeholders. *Allergy* **2008**, *63*, 597-609.

(79) Poms, R. E.; Klein, C. L.; Anklam, E. Methods for allergen analysis in food: a review. *Food Addit. Contam.* **2004**, *21*, 1-31.

(80) Morisset, M.; Moneret-Vautrin, D. A.; Kanny, G.; Guénard, L.; Beaudouin, E.; Flabbée, J.; Hatahet, R. Thresholds of clinical reactivity to milk, egg, peanut and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled oral challenges. *Clin. Exp. Allergy.* **2003**, *33*, 1046-1051.

(81) FAL, Food Allergen Laboratory, Rethimno, Crete, Greece; http://www.foodallergenslab.com (Accessed: 19th January 2012).

(82) van Hengel, A. Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal. Bioanal. Chem.* **2007**, *389*, 111-118.

(83) Platteau, C.; Cucu, T.; de Meulenaer, B.; Devreese, B.; de Loose, M.; Taverniers, I. Effect of protein glycation in the presence or absence of wheat proteins on detection of soybean proteins by commercial ELISA. *Food Addit. Contam. A* **2011**, 28, 127-135.

(84) Cucu, T.; Platteau, C.; Taverniers, I.; Devreese, B.; de Loose, M.; de Meulenaer, B. ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Addit. Contam. A* **2011**, 28, 1-10.

(85) Holzhauser, T.; Stephan, O.; Vieths, S. Detection of Potentially Allergenic Hazelnut (*Corylus avellana*) Residues in Food: A Comparative Study with DNA PCR-ELISA and Protein Sandwich-ELISA. *J. Agric. Food Chem.* **2002**, *50*, 5808-5815.

(86) Blais, B. W.; Gaudreault, M.; Phillippe, L. M. Multiplex enzyme immunoassay system for the simultaneous detection of multiple allergens in foods. *Food Control* **2003**, *14*, 43-47.

(87) Garber, E.; Perry, J. Detection of hazelnuts and almonds using commercial ELISA test kits. *Anal. Bioanal. Chem.* **2010**, *396*, 1939-1945.

(88) Schubert-Ullrich, P.; Rudolf, J.; Ansari, P.; Galler, B.; Führer, M.; Molinelli, A.; Baumgartner, S. Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Anal. Bioanal. Chem.* **2009**, *395*, 69-81.

(89) Rejeb, S. B.; Abbott, M.; Davies, D.; Cléroux, C.; Delahaut, P. Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Addit. Contam.* **2005**, *22*, 709-715.

(90) Diaz-Amigo, C. Antibody-based detection methods: From theory to practice. In *Molecular biological and immunological techniques and applications for food chemists*; Popping, B., Diaz-Amigo, C., Hoenicke, K., Eds.; John Wiley & Sons, Inc.: Hoboken, New Jersey. 2010; pp 223-245.

(91) Scheibe, B.; Weiss, W.; Ruëff, F.; Przybilla, B.; Görg, A. Detection of trace amounts of hidden allergens: hazelnut and almond proteins in chocolate. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *756*, 229-237.

(92) Jonsson, H.; Eriksson, A.; Malmheden Yman, I. Detecting food allergens with a surface plasmon resonance immunoassay. In *Detecting allergens in food*. Koppelman. S. J., Hefle, S. L., Eds.; CRC Press: Boca Raton, FL, 2006.

(93) Mohammed, I.; Mullett, W. M.; Lai, E. P. C.; Yeung, J. M. Is biosensor a viable method for food allergen detection? *Anal. Chim. Acta* **2001**, *444*, 97-102.

(94) Yman, I. M.; Eriksson, A.; Johansson, M. A.; Hellenas, K. E. Food allergen detection with biosensor immunoassays. *J. AOAC Int.* **2006**, *89*, 856-861.

(95) Bremer, M. G. E. G.; Smits, N. G. E.; Haasnoot, W. Biosensor immunoassay for traces of hazelnut protein in olive oil. *Anal. Bioanal. Chem.* **2009**, *395*, 119-126.

(96) Monaci, L.; Visconti, A. Mass spectrometry-based proteomics methods for analysis of food allergens. *Trac-Trends Anal. Chem.* **2009**, *28*, 581-591.

(97) Harrer, A.; Egger, M.; Gadermaier, G.; Erler, A.; Hauser, M.; Ferreira, F.; Himly, M. Characterization of plant food allergens: An overview on physicochemical and immunological techniques. *Mol. Nut. Food Res*. **2010**, *54*, 93-112.

(98) Careri, M.; Costa, A.; Elviri, L.; Lagos, J. B.; Mangia, A.; Terenghi, M.; Cereti, A.; Garoffo, L. Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara h 3/4 for food control by liquid chromatography–tandem mass spectrometry. *Anal. Bioanal. Chem*. **2007**, *389*, 1901-1907.

(99) Chassaigne, H.; Nørgaard, J. V.; van Hengel, A. J. Proteomics-based approach to detect and identify major allergens in processed peanuts by capillary LC-Q-TOF (MS/MS). *J. Agric. Food Chem.* **2007**, *55*, 4461-4473.

(100) Shefcheck, K. J.; Musser, S. M. Confirmation of the Allergenic Peanut Protein, Ara h 1, in a Model Food Matrix Using Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). *J. Agric. Food Chem*. **2004**, *52*, 2785-2790.

(101) Shefcheck, K. J.; Callahan, J. H.; Musser, S. M. Confirmation of Peanut Protein Using Peptide Markers in Dark Chocolate Using Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS). *J. Agric. Food Chem*. **2006**, *54*, 7953-7959.

(102) Bignardi, C.; Elviri, L.; Penna, A.; Careri, M.; Mangia, A. Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J. Chromatogr. A* **2010**, *1217*, 7579-7585.

(103) Heick, J.; Fischer, M.; Pöpping, B. First screnning method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J. Chromatogr. A* **2011**, *1218*, 938-943.

(104) Platteau, C.; de Loose, M.; de Meulenaer, B.; Taverniers, I. Detection of allergenic ingredients using real-time PCR: A case study on hazelnut (*Corylus avellana*) and soy (*Glycine max*). *J. Agric. Food Chem.* **2011**, *59*, 10803-10814.

(105) Mafra, I.; Ferreira, I. M. P. L. V. O.; Oliveira, M. B. P. P. Food authentication by PCR-based methods. *Eur. Food Res. Technol.* **2008**, *227*, 649-665.

(106) Ehlert, A.; Demmel, A.; Hupfer, C.; Busch, U.; Engel, K.-H. Simultaneous detection of DNA from 10 food allergens by ligation-dependent probe amplification. *Food Addit. Contamin. A* **2009**, *26*, 409- 418.

(107) Köppel, R.; Dvorak, V.; Zimmerli, F.; Breitenmoser, A.; Eugster, A.; Waiblinger, H.-U. Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur. Food Res. Technol.* **2010**, *230*, 367-374.

(108) Hird, H.; Lloyd, J.; Goodier, R.; Brown, J.; Reece, P. Detection of peanut using real-time polymerase chain reaction. *Eur. Food Res. Technol.* **2003**, *217*, 265-268.

(109) Pafundo, S.; Gullì, M.; Marmiroli, N. Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. *Anal. Bioanal. Chem.* **2010**, *396*, 1831-1839.

(110) Scaravelli, E.; Brohée, M.; Marchelli, R.; van Hengel, A. Development of three real-time PCR assays to detect peanut allergen residue in processed food products. *Eur. Food Res. Technol.* **2008**, *227*, 857-869.

(111) Scaravelli, E.; Brohée, M.; Marchelli, R.; van Hengel, A. The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR. *Anal. Bioanal. Chem.* **2009**, *395*, 127-137.

(112) Stephan, O.; Vieths, S. Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *J. Agric. Food Chem.* **2004**, *52*, 3754-3760.

(113) Mustorp, S.; Engdahl-Axelsson, C.; Svensson, U.; Holck, A. Detection of celery (*Apium graveolens*), mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) and sesame (*Sesamum indicum*) in food by real-time PCR. *Eur. Food Res. Technol.* **2008**, *226*, 771-778.

(114) Galan, A. G.; Brohée, M.; Scaravelli, E.; van Hengel, A.; Chassaigne, H. Development of real-time PCR assays for the detection of lupine residues in food products. *Eur. Food Res. Technol.* **2010**, *230*, 597-608.

(115) Galan, A. M. G.; Brohée, M.; de Andrade Silva, E.; van Hengel, A. J.; Chassaigne, H. Development of a real-time PCR method for the simultaneous detection of soya and lupin mitochondrial DNA as markers for the presence of allergens in processed food. *Food Chem.* **2011**, *127*, 834-841.

(116) Schöringhumer, K.; Redl, G.; Cichna-Markl, M. Development and validation of a duplex Real-Time PCR method to simultaneously detect potentially allergenic sesame and hazelnut in food. *J. Agric. Food Chem.* **2009**, *57*, 2126-2134.

(117) Platteau, C.; de Loose, M.; de Meulenaer, B.; Taverniers, I. Quantitative detection of hazelnut (*Corylus avellana*) in cookies: ELISA versus real-time PCR. *J. Agric. Food Chem.* **2011**, *59*, 11395-11402.

(118) D'Andrea, M.; Coïsson, J. D.; Travaglia, F.; Garino, C.; Arlorio, M. Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (*Corylus avellana* L.)

in foods through calibration via plasmid reference standard. *J. Agric. Food Chem.* **2009**, *57*, 11201-11208.

(119) Piknová, L.; Pangallo, D.; Kuchta, T. A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. *Eur. Food Res. Technol.* **2008**, *226*, 1155-1158.

(120) Brežná, B.; Hudecová, L.; Kuchta, T. A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. *Eur. Food Res. Technol.* **2006**, *223*, 373-377.

(121) Brežná, B.; Piknová, L.; Kuchta, T. A novel real-time polymerase chain reaction method for the detection of macadamia nuts in food. *Eur. Food Res. Technol.* **2009**, *229*, 397-401.

(122) Brežná, B.; Kuchta, T. A novel real-time polymerase chain reaction method for the detection of pecan nuts in food. *Eur. Food Res. Technol.* **2008**, *226*, 1113-1118.

(123) Brežná, B.; Dudášová, H.; Kuchta, T. A novel real-time polymerase chain reaction method for the qualitative detection of pistachio in food. *Eur. Food Res. Technol.* **2008**, *228*, 197-203.

(124) Ehlert, A.; Hupfer, C.; Demmel, A.; Engel, K.-H.; Busch, U. Detection of cashew nut in foods by a specific real-time PCR method. *Food Anal. Meth.* **2008**, *1*, 136-143.

(125) Brežná, B.; Dudášová, H.; Kuchta, T. A novel real-time polymerase chain reaction method for the detection of Brazil nuts in food. *J. AOAC Int.* **2010**, *93*, 197-201.

(126) Röder, M.; Filbert, H.; Holzhauser, T. A novel, sensitive and specific real-time PCR for the detection of traces of allergenic Brazil nut (*Bertholletia excelsa*) in processed foods. *Anal. Bioanal. Chem*. **2010**, *398*, 2279-2288.

(127) Pafundo, S.; Gullì, M.; Marmiroli, N. SYBR® GreenER™ Real-time PCR to detect almond in traces in processed food. *Food Chem.* **2009**, *116*, 811-815.

(128) Röder, M.; Vieths, S.; Holzhauser, T. Sensitive and specific detection of potentially allergenic almond (*Prunus dulcis*) in complex food matrices by TaqMan® real-time polymerase chain reaction in comparison to commercially available protein-based enzyme-linked immunosorbent assay. *Anal. Chim. Acta* **2011**, *685*, 74-83.

(129) Costa, J.; Mafra, I.; Oliveira, M. B. P. P. High resolution melting analysis as a new approach for the detection of Pru du 5 almond allergen in foods (submitted).

EXPERIMENTAL PART

High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods

Food Chemistry, **2012**, *133*, 1062-1069

Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods

Food Research International, **2013**, *51*, pp 228-235

Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols

Food Chemistry (submitted)

Food Chemistry 133 (2012) 1062–1069

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods

Joana Costa, Isabel Mafra*, M. Beatriz P.P. Oliveira

REQUIMTE, Laboratório de Bromatologia e Hidrologia, Faculdade de Farmácia, Universidade do Porto, Portugal.

*Corresponding author: Tel: +351 222078902. Fax: +351 222003977. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

Almond is responsible for trigging adverse immune responses in allergic individuals, and since it is present in many processed food, it is considered as a potential hidden allergen. Here we propose a novel, simple and highly specific approach to detect almond in a wide range of processed foods. The method consists of a real-time PCR assay targeting the gene encoding for the Pru du 5 allergen in almond, using the fluorescent Evagreen® dye combined with high resolution melting (HRM) analysis. The new approach allowed the detection of trace amounts of almond down to the level of 0.005% (w/w) and was successfully applied to processed foods. HRM analysis increased the specificity of the assay and was effective in distinguishing almonds from other plant foods, including the closely related fruits from the Rosaceae family. It was demonstrated for the first time that HRM analysis can provide a powerful tool for the identification of allergens in foods.

Keywords: Food allergies, almond, allergen detection, real-time PCR, HRM analysis

INTRODUCTION

Food allergies are, by definition, adverse responses of the immune system to food proteins, mainly glycoproteins. These reactions are attributed to Immunoglobulin E (IgE) or non-IgE (cellular) mechanisms and they affect almost 3-4% of adult population and 6% of young children (Sicherer & Sampson, 2006). Tree nuts comprise one of the eight groups of potentially allergenic foods known to be responsible for almost 90% of human allergies caused by food ingestion. In recent years, their use in food also led to an increasing concern about the rising number of sensitised individuals to tree nuts and peanut, which has intensified in Europe and USA (Sathe, Sharma, & Roux, 2008). Presently, in order to protect allergic individuals from atypical immune reactions, the total avoidance of any allergen containing food is recommended. However, this is a hard task to achieve, since processed food products commonly contain all sorts of ingredients, including potential allergens (Poms, Klein, & Anklam, 2004). To safeguard sensitised individuals, legislation on food labelling has established clear guidelines in European Union (EU), from which the recent Directive 2007/68/EC requires 14 groups of allergenic food ingredients with mandatory labelling: cereals containing gluten, crustaceous, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame, sulphur dioxide and, more recently, lupine and molluscs.

Almond, taxonomically designated as Prunus dulcis or Amygdalus communis L., belongs to the Rosaceae family, subfamily of the Prunoideae and is genetically related to fruits such as peach, plum, apples and cherries (Chen et al., 2008; Shahidi, Zhong, Wijeratne, & Ho, 2008). In conjunction with almond consumption, numerous mild to severe immune reactions, including potentially fatal anaphylactic shocks, have been described in sensitised and allergic individuals (Chen et al., 2008; Tawde, Venkatesh, Wang, Teuber, Sathe, & Roux, 2006). Until now, eight groups of allergens have been identified in almond, comprising the following proteins: Pru du 1 (PR-10 proteins), Pru du 2 (thaumatin-like proteins), Pru du 2S albumin (lipid transfer proteins), Pru du 3 (lipid transfer proteins), Pru du 4 (profilins), Pru du 5 (60S acidic ribosomal protein P2), Pru du 6 (amandin - legumins) and Pru du γ-conglutin (vicilins) (Costa, Mafra, & Oliveira, submitted for publication).

Pru du 5, also known as 60S acidic ribosomal protein P2, is encoded by the Prunus dulcis 60S acidic ribosomal protein gene (AL60SRP) (http://www.ncbi.nlm.nih.gov/nuccore/AY081851.1), has a sequence of 113 amino acids and a calculated molecular mass of 11.4 kDa, being recently reported as an almond allergen (Abolhassani & Roux, 2009). The presence of IgE antibodies for 60S acidic ribosomal protein P2 in 50% of almond sensitised patient's sera seems to classify Pru du 5 as a major allergen in almond, according to the allergen nomenclature guidelines specified by the International Union of

Immunological Societies (I.U.I.S.) Allergen Nomenclature Sub-committee (http://www.allergen.org).

To ensure compliance with the food labelling, reliable methods have been proposed for the detection and quantification of allergens in foods. Most of the commercially available and published techniques for allergen detection are based on the determination of potentially allergenic proteins by immunological assays (Albillos, Jin, Howard, Zhang, & Kothary, 2008; Kirsch, Fourdrilis, Dobson, Scippo, Maghuin-Rogister, & De Pauw, 2009; van Hengel, 2007), including some applications to almond detection (Garber & Perry, 2010; Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005; Roux, Teuber, Robotham, & Sathe, 2001; Scheibe, Weiss, Ruëff, Przybilla, & Görg, 2001). More recently, DNA-based methods have provided reliable tools of detecting hidden allergens in a wide range of foods (Mafra, Ferreira, & Oliveira, 2008; Monaci & Visconti, 2010). However, only a few polymerase chain reaction (PCR) applications have been reported for almond allergen detection (Köppel, Dvorak, Zimmerli, Breitenmoser, Eugster, & Waiblinger, 2010; Pafundo, Gullì, & Marmiroli, 2009; Pafundo, Gullì, & Marmiroli, 2010).

The novel approach of high-resolution melting (HRM) analysis emerged with the recent advances in high resolution instrumentation and with the specialised fluorescent DNA-binding dyes (Herrmann, Durtschi, Voelkerding, & Wittwer, 2006; Reed, Kent, & Wittwer, 2007). The method involves the gradual denaturation (melting) of PCR amplicons and detection of subsequent subtle fluorescent changes by the so-called new generation dyes present in the amplification reaction, such as LC Green PLUS (Idaho Technologies Ltd), Evagreen (Biotium) and SYTO9 (Invitrogen). The advantage of the new dyes over SYBR Green I is related to the possibility of using higher concentrations, generating greater fluorescent signals and increased sensitivity without causing PCR inhibition. HRM analysis offers a rapid high-throughput and cost-effective method that has been applied to genotyping, mutation scanning (Herrmann et al., 2006; Reed et al., 2007) and RNA editing (Chateigner-Boutin & Small, 2007). Applications of HRM for the development of single nucleotide polymorphism of almond cultivars (Wu et al., 2009; Wu, Wirthensohn, Hunt, Gibson, & Sedgley, 2008), for the verification of grapevine and olive cultivars using microsatellite markers (Mackay, Wright, & Bonfiglioli, 2008) and for the authentication of berry species using DNA barcodes (Jaakola, Suokasa, & Häggmana, 2010) have also been recently described.

In this work, we propose the application of HRM analysis as a highly specific and sensitive approach to detect trace amounts of almond in foods, targeting the AL60SRP gene encoding for the Pru du 5 allergen.

MATERIAL AND METHODS

Sample preparation Almond kernels were obtained from selected regional ("Casa Nova", "Duro Italiano", "Marcelina", "Orelha de mula", "Pegarinhos", "Refego", and "Verdeal") and commercial cultivars ("Ferradual", "Ferragnes", "Ferrastar", "Gloriette", and "Marcona"), collected in orchards located in Southwest region of Trás-os-Montes, Northeast of Portugal. Walnut kernels were collect from an orchard situated in Beira Alta region, Eastern of Portugal. Other tree nuts (walnut, macadamia nut, hazelnut, pine nut, Brazil nut, chestnut, cashew and pistachio), fruits from the Rosaceae family (peach, nectarine, apricot, cherry and plum) and other plant foods (peanuts, soybean, lupine, fava bean, maize, oat, barley, rice, pumpkin seeds, rapeseed, sunflower and tomato) were obtained in local markets. Processed foods (30 samples) containing almond and/or other tree nuts were also acquired at local markets, including nut snacks (3), cereal foods (3), biscuits (2), cake (1) and chocolate bars (21). All samples were ground and homogenised using a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany), separately using different material and different containers formerly treated with DNA decontamination solution.

Almonds and walnuts kernels, previously triturated, were combined using the laboratory knife mill to prepare model mixtures as binary reference standards containing 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5% and 10% of almond in walnut.

All food samples and binary reference mixtures were immediately stored at -20 ºC after preparation until DNA extraction.

DNA extraction

DNA from all samples was extracted using Nucleospin® Food kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions with minor modifications. To 200 mg of each sample, 700 µL of CF lysis solution pre-heated at 65 ºC and 10 µL of proteinase K (20 mg/mL) were added. The mixture was incubated in a thermal block (Thermomixer Compact, Eppendorf AG, Hamburg, Germany) at 65 ºC for 1 h with continuous stirring and centrifuged for 10 min (18,500g, 4 ºC). The supernatant was transferred (approximately 600 µL) to a new sterile reaction tube and the same volume of precipitation solution C4 and ethanol 100% were added. The mixture was homogenised by inversion and eluted through a spin column by centrifugation (1 min, 13,000g). The column was then washed three times: the first wash with 400 µL of CQW solution, the second and third washes with 700 µL and 200 µL of C5 solution, respectively, followed by 1 min centrifugation (13,000g) after the first two washings and a 2 min centrifugation after the final one. The DNA was eluted from the column by adding 100 µL of CE solution at 70 ºC, followed by a 5 min incubation period and centrifugation (1 min, 13,000g). All the extracts were kept at -20 ºC until further analysis. The extractions were done in duplicate assays for each sample.

Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometry using a spectrophotometer UV1800 Shimadzu (Kyoto, Japan). The DNA concentration was determined by UV absorbance at 260 nm (1 absorbance unit corresponds to 50 µg/mL of dsDNA). The purity of the extracted DNA was determined by the ratio of the absorbance at 260 and 280 nm.

Target gene selection and oligonucleotide primers

The DNA sequence corresponding to the gene encoding for the allergenic 60S acidic ribosomal protein of Prunus dulcis was retrieved from the Genbank database (accession number DQ836316). Specific primers Prd5-1F (GGT TGT TGC AGC ATA CTT GTT GGC) and Prd5-1R (GCT CCA ACA GAG CCA AGG ATG TCC) to produce a DNA fragment of 90 bp, were designed using the software Primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The nucleotide sequence was submitted to a basic local alignment search tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which identifies regions of local similarity among homologue sequences from different species and calculates the statistical significance of the matches. The programme confirmed the specificity of the designed primers for the selected AL60SRP gene sequence with 100% identity. The program did not find homology with any other sequence from different plant species. The primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

End-point PCR

Amplifications by end-point PCR were performed in 25 µL of total reaction volume containing 2 µL of DNA extract (100 ng), 15 mmol/L of Tris–HCl (pH 8.3), 50 mmol/L of KCl, 200 nmol/L of each primer Prd5-1F/Prd5-1R, 200 µmol/L of each dNTP (Invitrogen, Carlsbad, CA, USA), 3.5 mmol/L of MgCl2 and 1.5 U of DNA polymerase AmpliTaq Gold® (Applied Biosystems, Branchburg, NJ, USA). The reactions were performed in a thermal cycler MJ Mini (Bio-Rad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95 ºC for 5 min, with 40 cycles at 95 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 30 s, and a final extension at 72 ºC for 5 min.

The amplified fragments were analysed by electrophoresis in a 2.0% agarose gel containing Gel Red 1x (Biotium, Hayward, CA, USA) for staining and carried out in TAE buffer (40 mmol/L Tris–acetate, 1 mmol/L EDTA) for 60 min at 120 V. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital

Science™ DC120 (Rochester, NY, USA). Each extract was amplified at least in duplicate assays.

Real-time PCR and HRM analysis

The amplifications by real-time PCR were carried out in 20 µL of total reaction volume containing 2 µL of DNA extract of almond mixtures (50 ng) or commercial samples (100 ng), 1x of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 200 nmol/L of each primer Prd5-1F/Prd5-1R. The real-time PCR assays were performed on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 95 ºC for 5 min; 50 cycles at 95 ºC for 10 s and 66.6 ºC for 30 s, with collection of fluorescence signal at the end of each cycle. Data were collected and processed using the software Bio-Rad CFX Manager 2.0 (Bio-Rad Laboratories, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95 ºC for 1 min and then annealed at 65 ºC for 5 min in order to allow the correct annealing of the DNA duplexes. These two steps were followed by melting curve ranging from 65 to 95 ºC with temperature increments of 0.2 ºC every 10 s. The fluorescence data were acquired by the end of each melting temperature.

The collected fluorescence data were processed using the Precision Melt Analysis Software 1.0 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for easier visual identification of clusters. Cluster detection settings were defined targeting high sensitivity and threshold yields for more heterozygote clusters. Melting curve shape sensitivity determines the stringency used to classify melting curves into different clusters, thus high percentage value for this parameter allows increasing stringency and present results in more heterozygote clusters. Temperature of melting (Tm) difference threshold is a parameter that determines the lowest amount of Tm difference between samples and enables the inclusion of samples into different clusters. Therefore, melting curve shape sensitivity parameter was adjusted to percentage value >65% and Tm difference threshold parameter was set as default value of 0.15. Reference mixtures and samples were analysed in replicates (n=4) in two independent assays.

Sequencing of PCR products

To confirm the identity of the PCR products obtained with the Prd5-1F/Prd5-1R primers for almond and related species, the DNA fragments were sequenced. PCR products of peach, nectarine, apricot and two varieties of almond were purified with Cut&Spin DNA gel extraction columns (GRISP Research Solutions, Porto, Portugal) to remove interfering components. The purified products were sent to a specialized research facility (STABVIDA, Lisbon, Portugal) for sequencing. Each target fragment was sequenced twice performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with good quality.

RESULTS AND DISCUSSION

End-point PCR amplification

To specifically detect almond, the gene AL60SRP coding for Pru du 5 allergen was chosen from the available Prunus dulcis DNA/mRNA sequences in the Genbank database of NCBI. The choice was made because this protein was recently considered a potential major allergen for almond (Abolhassani & Roux, 2009) and based on the absence of homologue sequences in other plant sources. By using the primer-BLAST tool when designing the primers, it was possible to verify their 100% specificity towards the AL60sRP gene and lack of sequence similarity with other food species.

The results of estimating DNA concentration show that the extracts had, generally, high yields for all reference mixtures (403-596 ng/μL) and for the majority of the food samples (48-1253 ng/μL) under study. Complex food matrices such as chocolates also presented DNA extracts with adequate concentration range (15-130 ng/μL). The purity of extracts was generally close to 1.8 for all the reference mixtures and most foods, except for chocolates that ranged from 1.0 to 2.0.

Prior to the specific amplification of almond, all food samples were evaluated for their amplifiability with universal eukaryotic primers 18SEUDIR/18SEUINV (Fajardo, Gonzalez, Martin, Rojas, & Hernandez, 2008). All samples tested positively with the universal primers, confirming the absence of false negative results that might occur due to PCR inhibition.

To test and optimise the PCR conditions with the new primers Prd5-1F/Prd5-1R, DNA extracts from binary reference mixtures containing known amounts of almond in walnut were used. The amplification results enabled detecting the addition of almond down to the level of 0.005% (w/w), with no amplification observed for the 100% walnut kernels used to prepare the binary mixtures (data not shown).

To extensively test specificity, DNA from a wide range of plant species used for food was isolated. Table 1 presents the results of almond cultivars and several plant species tested for reactivity with primers Prd5-1F/Prd5-1R. The twelve almond cultivars tested positively for the target AL60SRP sequence confirming the specificity for almond species. Regarding the other eight commercial nuts analysed, walnut, hazelnut and macadamia nut produced some unspecific bands of different lengths, but none with 90 bp of the expected fragment. Peanut, soybean and fava bean also presented similar unspecific bands when tested by end-point PCR (Table 1).

All the remaining species tested negatively for PCR amplification, except the four samples of *Prunus persica* and *Prunus armeniaca*, which produced PCR fragments with the same size. This can be explained due to the genetic proximity of almond and other species that belong to the same genus. Genetic relation between almond and other Prunus fruits, like peach (Chen et al., 2008) or cherry (Dall'Antonia, Pavkov, Fuchs, Breiteneder, & Kellera, 2005), suspected to cause cross-reactivity, has already been demonstrated.

Since a new allergen from tomato was recently identified and included in the same protein family of Pru du 5 (López-Matas et al., 2011), this species was also tested in the present work. Tomato ARP60S exhibited high identity (80.7%) and homology (93.8%) with almond 60S acidic ribosomal protein (Pru du 5), which might contribute to possible cross-reactivity between these two fruits (López-Matas et al., 2011). However, in the present study, no PCR fragment was observed for tomato fruit, confirming the absence of cross-reactivity for this species (Table 1).

The applicability of the proposed sequence-specific primers to detect AL60SRP gene was further tested in a wide range of processed foods commercially available (Table 2). From a total of 30 food samples, eighteen out of the 25 samples labelled as containing almond tested positively for AL60SRP gene, suggesting the effectiveness of the technique in processed foods. The seven samples with negative amplification declared traces of almond or nut mixtures (containing almond), probably as a precautionary labelling to safeguard any possible cross-contamination at the production facility, and not because they really contained almond. Thus, their negative results might indicate the absence of almond contamination during the production of those food samples. From the five food samples with no allegation for almond, a sample of walnut cake gave a strong positive band of the expected PCR fragment of 90 bp (Table 2), suggesting the presence of unlabelled almond in this product.

Real-time PCR amplification

To verify the specificity of the proposed primers (Prd5-1F/Prd5-1R) and their applicability to real-time PCR amplification using the fluorescent dye Evagreen®, the DNA extracts of binary mixtures containing known amounts of almond (10%, 5%, 0.5%, 0.05%, 0.01% and 0.005%) were also used. A relative limit of detection and quantification of 0.005%, with high PCR efficiency (95.3%) and correlation (R2=0.972) was obtained with real-time PCR (Fig. 1a), confirming the previous results of end-point PCR.

Table 1. Results of PCR amplification of AL60SRP gene and HRM analysis applied to 12 almond cultivars and other plant species

Name	Scientific denomination	Country of Origin	End-point PCR	HRM analysis[b]
Almond “Pegarinhos” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (99.4%)
Almond “Orelha de mula” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (96.4%)
Almond “Verdeal” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (98.1%)
Almond “Duro Italiano” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (98.0%)
Almond “Ferragnes” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (85.1%)
Almond “Ferrastar” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (99.4%)
Almond “Ferraduel” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (97.3%)
Almond “Casa Nova” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (95.1%)
Almond “Gloriette” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (97.8%)
Almond “Refego” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (98.5%)
Almond “Marcelina” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (98.2%)
Almond “Marcona” cultivar	*Prunus dulcis*	Portugal	+	Cluster 1 (98.3%)
Walnut kernel	*Juglans regia*	Portugal	–	NA
Walnut[a]	*Juglans regia*	France	UA	Cluster 3 (100.0%)[c]
Macadamia nut[a]	*Macadamia tetraphylla*	Austria	UA	Cluster 2 (99.8%)[c]
Hazelnut	*Corylus avellana*	Portugal	UA	Cluster 4 (99.9%)[c]
Pine nut	*Pinus pinea*	Portugal	–	NA
Brazil nut[a]	*Bertholletia excelsa*	Bolivia	–	NA
Pistachio[a]	*Pistacia vera*	USA	–	NA
Cashew[a]	*Anacardium occidentale*	India	–	NA
Chestnut	*Castanea sativa*	Portugal	–	NA
Lupine	*Lupinus albus*	Portugal	–	NA
Fava bean[a]	*Vicia faba*	Greece	UA	ND
Peanut	*Arachis hypogaea*	USA	UA	Cluster 6 (89.4%)
Soybean	*Glycine max*	USA	UA	ND
Maize	*Zea mays*	USA	–	NA
Wheat	*Triticum aestivum*	Portugal	–	NA
Rice	*Oryza sativa*	Portugal	–	NA
Oat	*Avena sativa*	Portugal	–	NA
Barley	*Hordeum vulgare*	Spain	–	NA
Pumpkins seeds[a]	*Cucurbita mixta*	Greece	–	NA
Rapeseed	*Brassica napus*	Brazil	–	NA
Sunflower	*Helianthus annuus*	Brazil	–	NA
Rye	*Secale cereale*	Portugal	–	NA
Tomato	*Solanum lycopersicum*	Spain	–	NA
Peach	*Prunus persica*	Portugal	+	Cluster 5 (97.3%)
Nectarine	*Prunus persica*	Spain	+	Cluster 4 (82.7%)
Cherry	*Prunus avium*	Chile	–	NA
Plum	*Prunus domestica*	Chile	–	NA
Prune	*Prunus spinosa*	France	–	NA
Cherry plum	*Prunus cerasifera*	South Africa	–	NA
Apricot	*Prunus armeniaca*	Turkey	+	Cluster 3 (75.5%)
Black Apricot	*Prunus armeniaca*	Turkey	+	Cluster 2 (94.6%)

[a]Declaration in product label “may contain traces of peanut or other nuts”. [b]Percentage of confidence according the reference cluster “cluster 1”. [c]Samples included in different set of real-time PCR runs, where almond standards and commercial food containing almond were defined as reference cluster 1 (Fig.4 and Table 2). UA, Unspecific amplification. NA, Not applied. ND, Not detected. +, Positive detection. –, Not detected.

The respective melting curve analysis revealed the amplification of similar products since they exhibited the same melting temperature of 81.20 ºC (Fig. 1b). The amplification of processed food samples by real-time PCR with Evagreen® dye was in good agreement with the former end-point PCR amplifications results (Table 2).

Table 2. Results of PCR amplification of AL60SRP gene and HRM analysis applied to commercial food samples

Food Samples	Almond declaration	End-point PCR	HRM analysis[c]
Tree nut snack	+[a]	++	Cluster 1 (99.9%)
Tree nut and fruit snack	+[a]	++	Cluster 1 (99.6%)
Almond cereals	+[a]	++	Cluster 1 (99.7%)
Cereal bars with almond and chocolate	+	++	Cluster 1 (99.9%)
Cereal bars with almond and apricot	+	++	Cluster 1 (99.7%)
Walnut cake	–	++	Cluster 1 (99.9%)
Almond biscuits	+	++	Cluster 1 (99.9%)
Nougat and honey chocolate	+	+	Cluster 1 (99.9%)
Nougat chocolate	+[a]	++	Cluster 1 (99.3%)
Milk fruit and nut chocolate	+[a]	+	Cluster 1 (99.8%)
Milk chocolate with almonds 1	+[a]	+	Cluster 1 (98.9%)
Milk chocolate with almonds 2	+[a]	+	Cluster 1 (99.8%)
Milk chocolate with almonds 3	+[a]	+	Cluster 1 (99.7%)
Milk chocolate with almonds 4	+[a]	++	Cluster 1 (100.0%)
Milk chocolate with almonds 5	+[a]	++	Cluster 1 (99.5%)
Milk chocolate with caramelised almonds	+[a]	++	Cluster 1 (100.0%)
Milk chocolate with almonds, hazelnuts and raisins	+[a]	++	Cluster 1 (99.9%)
Chocolate with marzipan and strawberry	+[a]	++	Cluster 1 (99.5%)
White chocolate with hazelnuts	–	UA	Cluster 2 (98.8%)
Chocolate crispy nuts (hazelnuts)	–a	–	NA
Milk chocolate with hazelnuts	–a	–	NA
Milk chocolate with hazelnut cream	–a	–	NA
Black chocolate with nuts 1	+[a,b]	–	NA
Black chocolate with nuts 2	+[a,b]	–	NA
Black chocolate with nuts 3	+[a,b]	–	NA
Honey chocolate with nuts	+[a,b]	–	NA
Honey chocolate with fruits and nuts	+[a,b]	–	NA
Honey chocolate with nuts and crispy rice	+[a,b]	–	NA
Chocolate salami	+[a,b]	–	NA
Cookies with chocolate and nuts	–	–	NA

[a]Declaration "may contain traces of peanut or other nuts". [b]"may contain traces of almonds". [c]Percentage of confidence according the reference cluster "cluster 1". UA, Unspecific amplification. NA, Not applied. +, Faint to moderate bands. ++, Strong bands. –, Not detected.

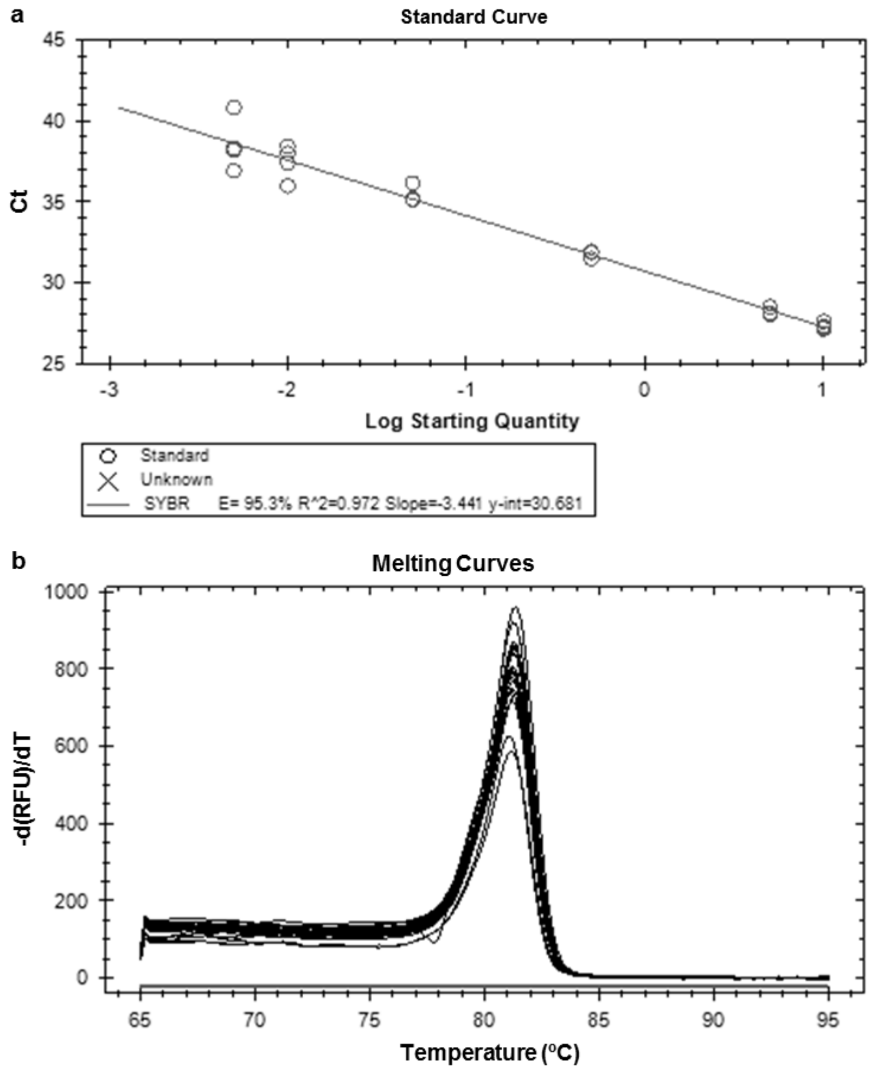


Fig. 1. Standard curve (a) and melting curve analysis (b) obtained by real-time PCR with Evagreen® dye amplification targeting the Pru du 5 gene of almond and applied to reference mixtures containing 10%, 5%, 0.5%, 0.05%, 0.01% and 0.005% of almond.

The melting curve analysis confirmed the specificity of the resultant products since all samples previously positive to almond produced fragments with the same melting temperature (Fig. 2). The unspecific amplifications for commercial walnut, macadamia nut and hazelnut (Table 1) were confirmed as such because they produced fragments with considerably lower melting temperatures than 81.20 ºC (Fig. 2). The suspected presence of almond in the sample of walnut cake was reinforced by the result of melting curve analysis.

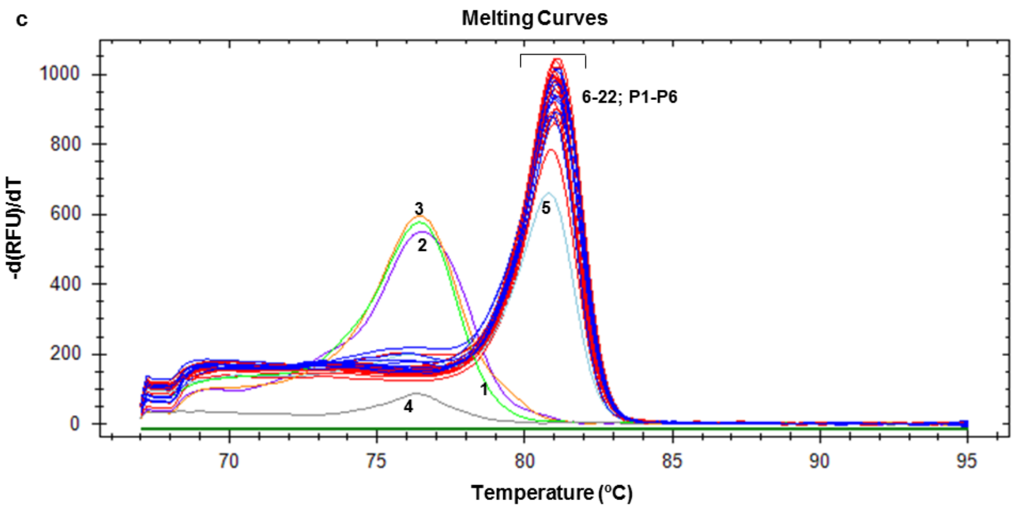


Fig. 2. Melting curves obtained by real-time PCR amplification with Evagreen® dye targeting the Pru du 5 gene of almond and applied to food samples. Legend: 1 – macadamia nut, 2 – hazelnut chocolate, 3 – hazelnut snack, 4 – walnut, 5 – walnut cake, 6-22 – processed foods (chocolates, cereals foods, nut mixes, biscuit), P1-P6 – 100% almond extracts serially diluted (4-fold).

HRM Analysis

HRM analysis takes advantage of the enhanced fluorescent dye and the possibility of gradually denature the PCR amplicons. Using an instrument with precise temperature control and homogeneity among the samples, slight sequence variations can be detected by denaturation of DNA fragments as a result of decreasing fluorescence due to dissociation of the double-stranded DNA-specific dye. Taking advantage of Evagreen® dye that is able to be used at higher concentrations than SYBR Green I, better sensitivity to sequence variation during melting can be achieved, whose changes are reflected in the differing shapes of the melting curves (Mackay et al., 2008). Small differences in the nucleotide sequence of real-time PCR fragments with similar size are sufficient to alter the form of the melting curve and the melting temperature (Jaakola et al., 2010). Different base substitutions can create minor variations in the melting behaviour and the resolution of those melting alterations requires an appropriate intercalating dye such as Evagreen® (Krypuy, Newnham, Thomas, Conron, & Dobrovic, 2006), which was chosen for the present study.

To further confirm the results obtained by end-point PCR and real-time PCR with Evagreen® dye, the novel approach of HRM analysis was investigated with the designated new primers. After real-time PCR amplification and melting curve analysis, all data were further analysed using the Precision Melt Analysis Software. Twelve almond cultivars were analysed together with six other species that amplified positively with real-time PCR (Fig. 3), while almond reference mixtures were tested with all the processed food samples (Fig. 4). In both cases, almond curves were included in the same cluster

(Cluster 1) and defined as the reference cluster, with percentages of confidence over 85% (Table 1, 2). The small differences among all melting curves presented in normalised plot (Fig. 3a) can be best visualised in the difference curve charts (Fig. 3b, 3c), while the great differences (Fig. 4a) are reproduced in the respective difference plot (Fig. 4b).

Fig. 3 shows the application of HRM analysis to individual genotypes of the same species (12 almond cultivars) and to six other species that amplified positively with real-time PCR. In Fig. 3b and 3c, it is possible to observe that the almond curves with similar shape were included in the same group, whereas the other Prunus fruits, namely black apricot, apricot, nectarine and peach, that formerly produced fragments with similar melting picks to almond (81.0-81.2 ºC) (data not shown), were distinguished and included in clusters from 2 to 5 (Table 1). Although these species produced fragments with similar size and melting temperature due to their genetic proximity, with the application of HRM analysis that is able to distinguish small variations among closely related species, they could be discriminated from almond. This finding agrees with results of application of HRM analysis to identify berry species (Jaakola et al., 2010), grapevine and olive cultivars (Mackay et al., 2008).

The application of HRM analysis to the second set of real-time PCR data of processed foods, almond reference mixtures and other nuts (Fig. 2) confirmed and reinforced their discrimination by both normalised melting curves (Fig. 4a) and difference curves (Fig. 4b). Almond reference mixtures and all the processed samples with previous positive amplification for almond were grouped in cluster 1 with high percentage of confidence, 98.9-100.0% (Table 2). Macadamia nut, walnut and hazelnut were included in different clusters: 2 to 4 with percentage of confidence of 99.8%, 100.0% and 99.9%, respectively (Fig. 4a, 4b, Table 1). Peanut was included in a different cluster (6) with 89.4% of confidence (Fig. 3b, Table 1).

The sample of walnut cake was included in the cluster 1 with 99.9% of confidence (Fig. 4), which was in good agreement with previous PCR results (Table 2) and melting curve analysis (Fig. 2), confirming the presence of unlabelled almond.

Sequencing results

The results presented by HRM analysis were investigated and confirmed by sequencing the PCR products from almond and other fruits from the Rosaceae family. The sequencing results revealed identical nucleotide sequences of 91 bp (100% identity) for both almond varieties. In comparison with the available Genbank sequence, the obtained sequences exhibited one A base deletion at 58 position and insertions of 2 A bases in position 89 and 91 from 5’end of the strand.

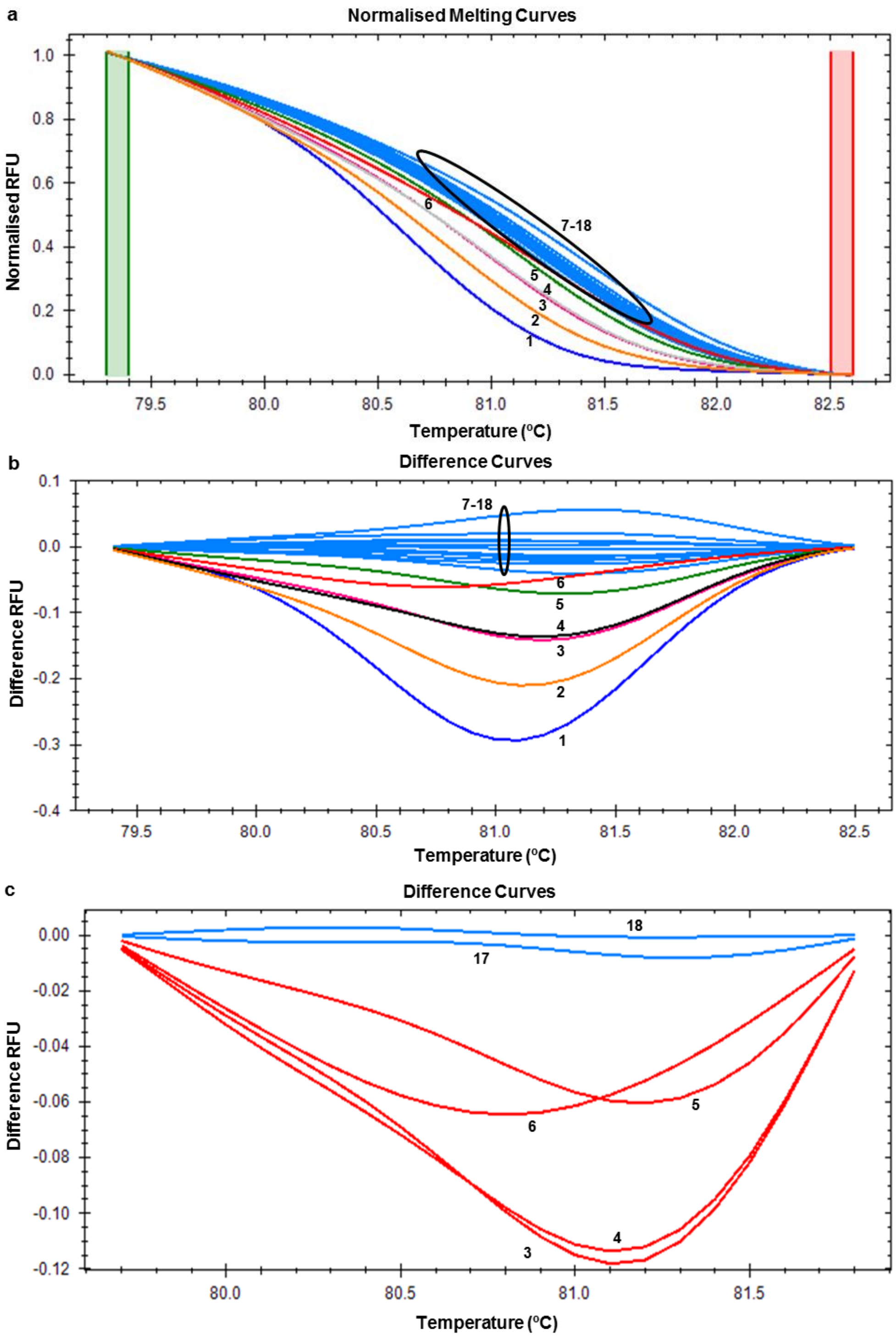


Fig. 3. HRM analysis of real-time PCR products with Evagreen® dye targeting the Pru du 5 gene of almond and applied to different almond cultivars and other plant species. (a) Normalised melting curves and (b, c) difference curves. Legend: 1 – Hazelnut, 2 – Peanut, 3 – Apricot, 4 – Nectarine, 5 – Black Apricot, 6 – Peach, 7-18 – almond cultivars produced in Portugal, namely "Orelha de mula", "Pegarinhos", "Ferrestar", "Ferragnes", "Ferraduel", "Gloriette", "Casa Nova", "Refego", "Marcelina", "Marcona", "Duro Italiano" and "Verdeal", respectively.

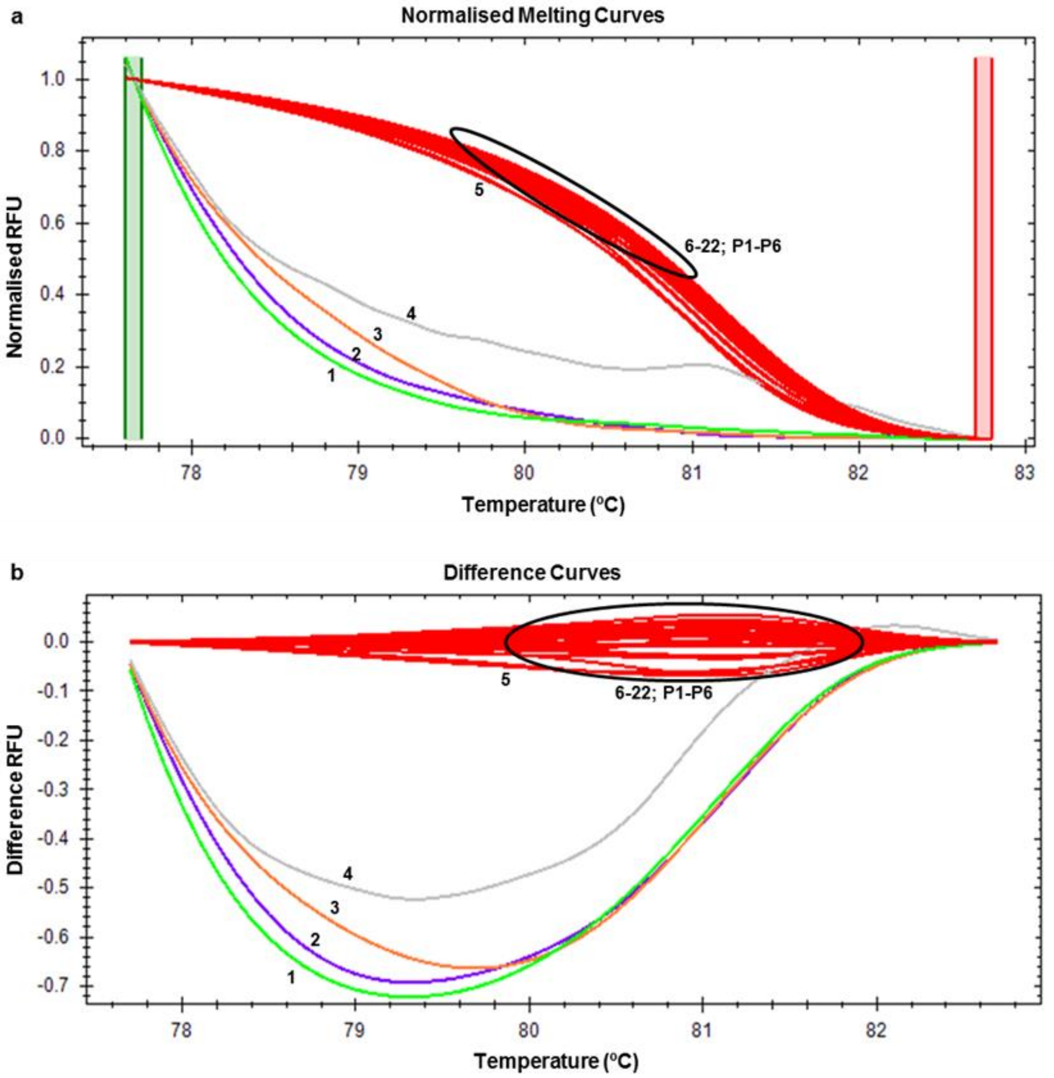


Fig. 4. HRM analysis of real-time PCR products with Evagreen® dye targeting the Pru du 5 gene of almond and applied to different processed food samples and almond DNA extracts. (a) Normalised melting curves and (b) difference curves. Legend: 1 – Macadamia nut, 2 – hazelnut chocolate, 3 – roasted hazelnut, 4 – commercial walnut, 5 – walnut cake, 6-22 – Commercial samples containing almond (chocolates, salami, cereals, cereal bars, nuts mixes), P1-P6 – Reference standards containing 100% of almond serially diluted (4-fold).

Sequenced fragments of nectarine, peach and apricot, that revealed similar sizes as expected (90 bp for the former and 91 bp for the others), were compared with the achieved almond sequence. Nectarine nucleotide sequence presented a mismatch (G → T) in position 4, deletion of T and A bases from positions 7 and 90, respectively, and an insertion of N base in position 9 from the 5’ end. Peach sequence evidenced a mismatch (T → G) in position 1 and an insertion of G base in position 11 from the 5’ end. Apricot sequence revealed insertion of a T base in position 2 and a deletion of a A base from position 89. Although the differences between almond and the other Prunus sequences are small, they constitute the reason for HRM analysis to exclude peach, nectarine and apricot from the same cluster of almond. Since HRM analysis allow discriminating DNA sequences based on small variations such as a single nucleotide mismatch, sequencing of Prunus fruits enabled to confirm and reinforce the results from the HRM analysis.

CONCLUSIONS

The present work has demonstrated the usefulness of the designed new primers to detect a specific DNA region encoding the allergenic protein Pru du 5 in almond. The optimised conditions for both end-point PCR and real-time PCR were successfully achieved with the designated primers down to the level of 0.005% of almond addition, obtaining adequate PCR efficiency and correlation with real-time PCR. The proposed assays were also effective when applied to real processed food samples and to other non-related and related plant species. Regarding related species of Prunus genus such as peach, nectarine and apricot, similar fragments were identified by both end-point PCR and real-time PCR with similar melting temperatures. However, with HRM analysis it was possible to discriminate almond from other Prunus fruits. HRM analysis was able to demonstrate that the unspecific PCR products obtained for commercial walnut, macadamia nut and hazelnut were distinguished from almond amplification. HRM analysis also enabled the unequivocal identification of the AL60SRP gene present in commercial foods containing almond and the non-compliance with the labelling statement in one sample of walnut cake, which can represent a risk for allergic individuals.

In summary, we have proposed a novel and simple approach to detect almond allergens in processed foods by the use of end-point PCR and real-time PCR with Evagreen® dye. In addition to the simple and sensitive real-time PCR assay, we apply for the first time HRM analysis as a cost-effective and powerful tool for high-throughput identification of allergens in foods.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

The authors have declared no conflict of interest.

REFERENCES

Abolhassani, M., & Roux, K. H. (2009). cDNA cloning, expression and characterization of an allergenic 60s ribosomal protein of almond (Prunus dulcis). Iranian Journal of Allergy, Asthma & Immunology, 8, 77–84.

Albillos, S. M., Jin, T., Howard, A., Zhang, Y., Kothary, M. H., & Fu, T.-J. (2008). Purification, crystallization and preliminary X-ray characterization of Prunin-1, a major component of the almond (Prunus dulcis) allergen amandin. Journal of Agriculture and Food Chemistry, 56, 5352–5358.

Chateigner-Boutin, A.-L., & Small, I. (2007). A rapid high-throughput method for the detection and quantification of RNA editing based on high-resolution melting of amplicons. Nucleic Acids Research, 35, e114. Doi:10.1093/nar/gkm640.

Chen, L., Zhang, S., Illa, E., Song, L., Wu, S., Howad, W., et al. (2008). Genomic characterization of putative allergen genes in peach/almond and their synteny with apple. BMC Genomics, 9 (1), 543.

Costa, J., Mafra, I., & Oliveira, M. B. P. P. (2011). Almond allergens: molecular characterization, detection and clinical relevance (submitted for publication).

Dall'Antonia, Y., Pavkov, T., Fuchs, H., Breiteneder, H., & Kellera, W. (2005). Crystallization and preliminary structure determination of the plant food allergen Pru av 2. Acta Crystallographica Section F Structural Biology and Crystallization Communications, F61, 186–188.

Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. Official Journal of European Union, L310, 11−14.

Fajardo, V., Gonzalez, I., Martin, I., Rojas, M., Hernandez, P. E., Garcia, T., et al. (2008). Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. Meat Science, 79, 289-298.

Garber, E., & Perry, J. (2010). Detection of hazelnuts and almonds using commercial ELISA test kits. Analytical and Bioanalytical Chemistry, 396, 1939–1945.

Herrmann, M. G., Durtschi, J. D., Voelkerding, K. V., & Wittwer, C. T. (2006). Instrument comparison for DNA genotyping by amplicon melting. Journal of the Association for Laboratory Automation, 11, 273–277.

Jaakola, L., Suokasa, M., & Häggmana, H. (2010). Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analyses of berry species. Food Chemistry, 123, 494–500.

Kirsch, S., Fourdrilis, S., Dobson, R., Scippo, M.-L., Maghuin-Rogister, G., & De Pauw, E. (2009). Quantitative methods for food allergens: a review. Analytical and Bioanalytical Chemistry, 395, 57–67.

Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A.; Eugster, A., & Waiblinger, H.-U. (2010). Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. European Food Research and Technology, 230, 367–374.

Krypuy, M., Newnham, G., Thomas, D., Conron, M., & Dobrovic, A. (2006). High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. BMC Cancer, 6, 295–306.

López-Matas, M. A., Ferrer, A., Larramendi, C. H., Huertas, A. J., Pagán, J. A., García-Abujeta, J. L., et al. (2011). Acidic ribosomal protein 60S: A new tomato allergen. Food Chemistry, 127, 638–640.

Mackay, J. F., Wright, C. D., & Bonfiglioli, R. G. (2008). A new approach to varietal identification in plants by microsatellite high resolution melting analysis: application to the verification of grapevine and olive cultivars. Plant Methods, 4, 8. Doi:10.1186/1746-4811-4-8

Mafra, I., Ferreira, I. M. P. L. V. O., & Oliveira, M. B. P. P. (2008). Food authentication by PCR-based methods. European Food Research and Technology, 227, 649–665.

Monaci, L., & Visconti, A. (2010). Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. Trends in Food Science and Technology, 21, 272–283.

Pafundo, S., Gullì, M., & Marmiroli, N. (2009). SYBR® GreenER™ Real-Time PCR to detect almond in traces in processed food. Food Chemistry, 116, 811–815.

Pafundo, S., Gullì, M., & Marmiroli, N. (2010). Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. Analytical and Bioanalytical Chemistry, 396, 1831–1839.

Poms, R. E., Klein, C. L., & Anklam, E. (2004). Methods for allergen analysis in food: a review. Food Additives and Contaminants, 21, 1–31.

Reed, G. H., Kent, J. O., & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics, 8, 597–606.

Rejeb, S. B., Abbott, M., Davies, D., Cléroux, C., & Delahaut, P. (2005). Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. Food Additives and Contaminants, 22, 709–715.

Roux, K. H., Teuber, S. S., Robotham, J. M., & Sathe, S. K. (2001). Detection and Stability of the Major Almond Allergen in Foods. Journal of Agriculture and Food Chemistry, 49, 2131–2136.

Sathe, S., Sharma, G., & Roux, K. (2008). Tree Nut Allergens. In C. Alasalvar, & F. Shahidi (Eds.), Tree Nuts: Composition, Phytochemicals, and Health Effects (pp. 65–83), Boca Raton, FL: CRC Press.

Scheibe, B., Weiss, W., Ruëff, F., Przybilla, B. & Görg, A. (2001). Detection of trace amounts of hidden allergens: hazelnut and almond proteins in chocolate. Journal of Chromatography B: Biomedical Sciences and Applications, 756, 229–237.

Shahidi, F., Zhong, Y., Wijeratne, S., & Ho, C.-T. (2008). Almond and Almond Products: Nutraceutical components and health effects. In C. Alasalvar, & F. Shahidi (Eds.), Tree Nuts: Composition, Phytochemicals, and Health Effects (pp. 127–141), Boca Raton, FL: CRC Press.

Sicherer, S. H., & Sampson, H. A. (2006). Food allergy. Journal of Allergy and Clinical Immunology, 117, S470–S475.

Tawde, P., Venkatesh, Y. P., Wang, F., Teuber, S. S., Sathe, S. K., & Roux, K. H. (2006). Cloning and characterization of profilin (Pru du 4), a cross-reactive almond (Prunus dulcis) allergen. Journal of Allergy and Clinical Immunology, 118, 915–922.

van Hengel, A. (2007). Food allergen detection methods and the challenge to protect food-allergic consumers. Analytical and Bioanalytical Chemistry, 389, 111–118.

Wu, S.-B., Wirthensohn, M., Hunt, P., Gibson, J., & Sedgley, M. (2008). High resolution melting analysis of almond SNPs derived from ESTs. Theoretical and Applied Genetics, 118, 1–14.

Wu, S.-B., Tavassolian, I., Rabiei, G., Hunt, P.; Wirthensohn, M., Gibson, J., et al. (2009). Mapping SNP-anchored genes using high-resolution melting analysis in almond. Molecular Genetics and Genomics, 282, 273–281.

Food Research International 51 (2013) 228–235

Contents lists available at SciVerse ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods

Joana Costa, Maria Beatriz P.P. Oliveira, Isabel Mafra*

REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal.

*Corresponding author: Tel: +351 220428640. Fax: +351 226093390. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

Almond is a widely consumed nut due to its pleasant flavour and health benefits. However, since this nut is commonly present in many processed foods, it is regarded as a potential hidden allergen. In this work, the novel single-tube nested real-time PCR system, already successfully applied to hazelnut and peanut detection, was attempted to trace almond allergens in foods. The method consists of using two sets of primers and a hydrolysis probe specifically designed to target *Prunus dulcis* clone 276NPL *prunin* gene that encodes the Pru du 6 allergen. The system allowed lowering the limit of detection of the conventional real-time PCR from 100 mg/kg to 50 mg/kg of almond spiked in walnut. It also enabled an absolute sensitivity of 1.28 pg of almond DNA that corresponds to about 3.9 DNA copies. These results highlight the suitability of the newly developed method for the detection of almond allergens in processed foods. To our knowledge, these findings were never reported and represent a great achievement when considering the detection of this almond allergen in food products.

Keywords: Almond detection, single-tube nested real-time PCR, tree nuts, food allergens

INTRODUCTION

Almond (*Prunus dulcis* or *Amygdalus communis* L.) belongs to the tree nut group and it is considered one of the most appreciated and consumed nuts. Especially due to the potential health benefits attributed to this nut and to its recent recognition by the Food and Drug Administration (FDA), as "heart-protective" foods (FDA, 2003), the consumption of almond and other nuts has been increasing over the past years. Thus, almond can be found in all sorts of product formulations ranging from bakery foods to chocolates or confectionary foodstuffs (Alasalvar & Shahidi, 2008), which in some cases can be present as a hidden ingredient. Although almond can be safely ingested by the majority of the population, it can also represent a problem of public health to a certain percentage of sensitised consumers since it is a source of allergenic proteins. Until now, eight groups of allergenic proteins have been characterised in almond, being responsible to induce mild to potentially fatal reactions in sensitised individuals (Costa, Mafra, Carrapatoso, & Oliveira, 2012).

The actual prevalence of allergies related to tree nuts is not yet well established, though recent studies seem to indicate a clear increase on the incidence of tree nut allergies among young population. In the USA, the prevalence of allergy in children raised from 0.6% to 2.1% between 1997 and 2008 (Sicherer, Muñoz-Furlong, Godbold, & Sampson, 2010). Within the tree nut group, almond, walnut, cashew, pecan and Brazil nut are known to be responsible for food related allergies in the USA (Sicherer & Sampson, 2006). In Europe, allergy to hazelnut consumption is more frequent and is often related to birch pollinosis (Burney et al., 2010). Therefore, it is mandatory to protect sensitised and allergic individuals from potentially life-threatening foods since the total avoidance of allergenic ingredients is rather difficult to achieve. In addition to this and mostly due to the excessively precautionary labelling practiced by food industry, allergic consumer's choices have become significantly restricted, affecting consequently their quality of life. Presently, food allergen management is considered as a major challenge, particularly to the clinical community and to the food industry (Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005). Therefore, the development of proper analytical methodologies to help industry managing the food allergens and to ensure consumer's safety is crucial.

Actually, most of the analytical tools used for the detection of food allergens target either proteins or DNA molecules (Holck et al., 2011). Regarding almond detection and quantification, some analytical approaches have been successfully applied. The immunochemical assays such as enzyme-linked immunosorbent assays (ELISA) or lateral flow devices (LFD) have been extensively used since they exhibited the advantage of directly detecting the offending ingredients (marker proteins) (Garber & Perry, 2010; Rejeb et al., 2005; Röder, Vieths, & Holzhauser, 2011; Scheibe, Weiss, Ruëff, Przybilla, & Görg, 2001). However, immunoassays present some major drawbacks that encompass the problems with the alteration of protein conformational structure as a result of food processing (van Hengel, 2007) or cross-reactivity with non-target proteins, especially with other fruits from the Rosaceae family. More recently, mass-spectrometry based techniques have also been successfully applied to the direct monitoring of almond proteins in food products (Bignardi, Elviri, Penna, Careri, & Mangia, 2010; Heick, Fischer, & Pöpping, 2011).

Considering the availability of alternative analytical techniques, the DNA-based methods have attained a special emphasis since DNA molecule present relatively high stability upon food processing. Even though these techniques are regarded as indirect approaches for the detection of allergenic ingredients, the methods based on polymerase chain reaction (PCR) have been effectively used to detect almond in foods with good specificity and sensitivity (Costa, Mafra, & Oliveira, 2012; Pafundo, Gullì, & Marmiroli, 2009; Pafundo, Gullì, & Marmiroli, 2010, Röder et al., 2011; Wang, Li, Zhao, Chen, & Ge, 2011). The referred studies present good sensitivity levels for almond detection, but only Pafundo et al. (2009 and 2010) used a system targeting amandin (Pru 1 or Pru du 6) in foods. The method reported by Pafundo et al. (2010) was based on real-time PCR with a non-sequence specific binding DNA molecule (SYBR® GreenER™), reaching adequate sensitivity of almond DNA encoding Pru du 6 allergen, nevertheless lacked cross-reactivity assessment with related or non-related species.

In terms of molecular characterisation, Pru du 6 belongs to the cupin superfamily and is the most well studied allergen in almond (Albillos, Menhart, & Fu, 2009; Sathe et al., 2002; Jin et al., 2009), probably because it is a storage protein present in high amounts in almond kernel. Additionally, it is classified as a major allergen that is responsible for triggering severe allergic reactions (potentially life-threatening) in sensitised individuals (Roux, Teuber, & Sathe, 2003), which emphasises the importance of using Pru du 6 as a target almond allergen.

The aim of this work was to apply for the first time the new approach based on single tube nested real-time PCR system for the detection and semi-quantification of almond allergens in food products. The developed method was based on the same principle as reported by Bergerová, Brežná, and Kuchta (2011) and Costa, Mafra, Kuchta, and Oliveira (2012) for peanut and hazelnut detection, respectively, assembling the advantages of two techniques, namely, nested PCR and real-time PCR in one single reaction. The nested PCR technique allows producing PCR fragments with two different sizes, increasing the specificity of the reaction. The use of a fluorogenic probe during the real-time PCR assay enables the direct monitoring of the fragment production throughout the entire second phase of the reaction. The application of this new system aimed at enhancing the sensitivity and specificity of almond DNA detection in foods. This study also intends to compare the conventional real-time PCR technique with the proposed single-tube nested real-time PCR method regarding almond detection.

MATERIALS AND METHODS

Plant foods and sample preparation

Almonds were obtained from selected regional cultivars (“Duro Italiano”, “Orelha de mula”, “Casa Nova”, “Pegarinhos”, “Refego”, “Marcelina” and “Verdeal”) collected in orchards located in Southwest region of Trás-os-Montes (Northeast of Portugal) and commercial cultivars (“Gloriette”, “Ferragnes”, “Ferrastar”, “Ferradual”, and “Marcona”). Almond and other tree nuts that included walnut, macadamia nut, hazelnut, Brazil nut, chestnut, pine nut, cashew, pistachio and peanut, as well as different plant foods (soybean, lupine, fava bean, maize, oat, barley, rice, rye, wheat, pumpkin seeds, rapeseed, sunflower, tomato, peach, apricot, plum and cherry) were obtained at local markets.

In the absence of certified or testing reference standards for almond, binary model mixtures containing 10 mg/kg; 50 mg/kg; 100 mg/kg; 500 mg/kg; 1,000 mg/kg; 5,000 mg/kg; 10,000 mg/kg; 50,000 mg/kg and 100,000 mg/kg of almond in walnut matrix were prepared. The first sample containing 100,000 mg/kg of almond (10%) was prepared by adding 20 g of almond to 180 g of walnut. All the other model mixtures were serially diluted by successive additions of walnut material until 10 mg/kg (0.001%) in the equivalent proportion.

All plant food samples, as well as reference mixtures were ground and homogenised separately, into a fine powder of approximately 0.3 mm of diameter in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) using different containers and material, previously treated with a DNA decontamination solution. To avoid accidental cross-contamination among samples, plant foods and standards, all materials were grounded in different days. The fruits, namely, tomato, peach, apricot, plum and cherry, were lyophilised before grinding.

After preparation, all samples and reference mixtures were immediately stored at -20 ºC until further DNA extraction.

DNA extraction

DNA was extracted from all samples by chaotropic solid-phase extraction using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations, as described by Costa et al. (2012c). Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometry using a spectrophotometer UV1800 Shimadzu (Kyoto, Japan).

Target gene selection and oligonucleotide primers

The DNA sequence corresponding to *Prunus dulcis* clone 276NPL *prunin* gene encoding for the Pru du 6 allergen was retrieved from the Genbank database (accession no. EU919663). Two sets of primers with different annealing temperatures (T_a) were designed using the software Primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The software parameters were set to design a first pair of primers (Prd6-1F/Prd6-1R) with an optimal T_a of 65 ºC. The second pair of primers was projected to have lower T_a (54 ºC), considering a difference of at least 10 ºC between the two sets of primers. For the application of real-time PCR systems a hydrolysis probe was also designed (Table 1).

Table 1. Key data of primers and probe designed to target *Prunus dulcis* clone 276NPL *prunin* gene partially encoding for Pru du 6 protein (GenBank accession no. EU919663).

Oligonucleotides	Sequence (5'-3')	Amplicon (bp)
Outer primers		
Prd6-1F	CCGCAGAACCAGTGCCAGCT	121
Prd6-1R	CCCCGGCACACTGGAAGTCCT	
Prd6-1P	FAM-GCTTCAAGCCCGCGAACCCGACAAC-BHQ2	
Inner primers		
Prd6-1FN	AACCAGTGCCAGCTCAA	88
Prd6-1RN	TGAAGTTCCAGGTCTCGAT	
Sequencing primers		
Prd6-FS	CTCCTTCTCATTCCTTGTGTAATGG	274
Prd6-RS	AGGAAGGCAAGTGTAGGCCGTT	

The basic local alignment search tool BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used to identify regions of local similarity between the chosen nucleotide sequence and homologue sequences of different species. In addition, the programme estimates the statistical significance of the matches, which confirmed the specificity of the designed primers for the selected *Prunus dulcis* clone 276NPL *prunin* gene (accession no. EU919663), *Prunus dulcis* prunin 1 precursor (accession no. GU059260) and *Prunus amygdalus* Batsch (Texas) pru1 mRNA (accession no. X78119) with 100%, 99% and 98% identity, respectively. Any other additional homology was found with plant species. Primers and probe were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Sequencing

For sequencing the region of interest, a third set of primers (Prd6-FS/Prd6-RS) was specifically designed to produce larger fragments with 274 bp, encompassing the target region of 121 bp amplified by the primers (Prd6-1F/Prd6-1R), using the end-point PCR conditions described below. The amplified fragments of five almond samples containing four different cultivars and the commercial almond used to prepare the model mixtures were sequenced. Due to the usual problems of cross-reactivity between almond, peach and apricot, and since these two samples presented positive end-point PCR bands, they were also sequenced. All PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components and sent to a specialised research facility (STABVIDA, Lisbon, Portugal) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality.

End-point PCR

PCR amplifications were carried out in 25 µL of total reaction volume containing 2 µL of almond DNA extract (100 ng), 670 mM of Tris–HCl (pH 8.8), 160 mM of $(NH_4)_2SO_4$, 0.1% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of $MgCl_2$ and 200 nM of each primer Prd6-FS/Prd6-RS (Table 1). The reactions were performed in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA) using the following programme: initial denaturation at 95 ºC for 5 min; 40 cycles at 95 ºC for 30 s, 65 ºC for 45 s and 72 ºC for 1 min; and a final extension at 72 ºC for 5 min.

Real-time PCR assays

Real-time PCR assays were performed in 20 µL of total reaction volume. For each reaction tube, 2 µL of DNA (100 ng), 1x of SsoFast Probes Supermix (Bio-Rad, Hercules, CA, USA), 300 nM of each outer primer Prd6-1F/Prd6-1R and 150 nM of hydrolysis probe Prd6-1P (Table 1). For nested real-time PCR amplification, the mix included additionally 300 nM of the primers Prd6-1FN/Prd6-1RN, specifically designed for this assay (Table 1). All real-time PCR assays were made on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Real-time PCR amplifications based on the conventional technique were performed according to the following temperature protocol: 95 ºC for 5 min, 50 cycles at 95 ºC for 15 s and 65 ºC for 45 s, with the fluorescence signal acquisition at the end of each cycle. Nested real-time PCR assays were carried out with two different programmes of temperature as presented in Table 2: phase 1, performed without collecting fluorescence signal; and phase 2, with collection of the fluorescence signal made at the end of each cycle. Data were collected and analysed using the software Bio-Rad CFX Manager 2.1 (Bio-Rad, Hercules, CA, USA). Cycle threshold (Ct) values were calculated using the software at automatic threshold setting. Real-time PCR and nested real-time PCR trials were repeated two or three times using three replicates, respectively.

Table 2. Temperature protocol for nested real-time PCR system.

	Phase 1			Phase 2		
	Initial denaturation	Denaturation	Annealing/ Polymerisation	Denaturation	Annealing	Polymerisation
Temperature	95 ºC	95 ºC	65 ºC	95 ºC	54 ºC	72 ºC
Time	5 min	15 s	45 s	15 s	20 s	30 s
Number of cycles		10 or 15		35 or 40		

RESULTS

For the proposed method, two sets of primers with different annealing temperatures were designed. The first set generating PCR fragments of 121 bp were used as the “outer” primers to delineate the chosen target sequence (Table 1, Fig. 1.). Thus, for this system, primers Prd6-1F/Prd6-1R and probe Prd6-1P were selected to hybridise at high temperatures (65 ºC), conferring great selectivity to the reaction. The second set of primers (Prd6-1FN/Prd6-1RN), producing PCR fragments of 88 bp, were defined to act as “inner” primers at lower hybridisation temperatures (54 ºC). The successful empirical rule for single-tube nested real-time PCR system, T_a (inner primers) < T_a (outer primers) < T_a (probe), used for Ara h 3 (Bergerová et al., 2011) and hsp1 (Costa et al., 2012c) detections, was also followed in this approach. In order to perform the single-tube nested real-time PCR system, two independent temperature phases have to be established (Table 2). During phase 1, PCR fragments of 121 bp were amplified to function as DNA template later on the reaction, with no fluorescence acquisition. Phase 2 was programmed to produce PCR fragments of 88 bp using the 121 bp fragments as template and the collection of fluorescence was performed at the end of each cycle. Therefore, the number of cycles used in each phase was defined considering the best performance in nested real-time PCR trials (data not shown). Phase 1 was set during 10 or 15 cycles, without fluorescence collection. In phase 2 fluorescence signal acquisition was carried out using 35 or 40 cycles, according to the initial number of cycles used in phase 1.

1 GGGGTGAGCTCGAATTACAAGTCAKCTATCTCCTTCTCATTCCTTGTGTAATGGCTARGG

61 CTTTCGTTTTTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGT

121 CCCAGTTGAGT**CCGCAGAACCAGTGCCAGCT**CAACCA**GCTTCAAGCCCGCGAACCCGACA**

181 **AC**CGCATCCAGGCKGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGG**AGGACTTCC**

241 **AGTGTGCCGGGG**TSGCCGCCTCTCGAATCACCATTCAGCGCAACGGCCTACACTTGCCTT

301 CCTACTCCAAC

Fig. 1. Region of the *Prunus dulcis* clone 276NPL *prunin* gene partially encoding the Pru du 6 allergen in almond (GenBank accession no. EU919663). "Outer" primers (Prd6-1F/Prd6-1R) in bold, "inner" primers (Prd6-1FN/Prd6-1RN) underlined and probe (Prd6-1P) in bold and double-underlined. Primers (Prd6-FS/Prd6-RS) used for sequencing are shaded in grey.

Sequencing of PCR products

An easy, simple and very reliable strategy for sequencing small regions is to use a third set of primers (Prd6-FS/Prd6-RS) to amplify a larger fragment (274 bp), encompassing the target sequence of 121 bp. This strategy was already successfully accomplished for the sequencing of hazelnut fragments in a previous report (Costa et al., 2012c). With this approach it was possible to amplify PCR fragments with adequate size for direct sequencing. The sequencing results for the confirmation of the identity of the PCR products are presented in Fig. 2. Regarding almond cultivars and the commercial sample used to prepare model standards, their sequences evidenced 100% homology with the sequence retrieved from GenBank (accession no. EU919663). However, the obtained sequences also highlighted the reasons for the existence of some cross-reactivity among species from the Rosaceae family. Though both apricot and peach DNA could be amplified with the designed primers, it was also possible to identify some differences between the sequences of almond and the other fruits (peach and apricot) in the target region of 121 bp. Peach and the respective variety of nectarine presented the same sequence, but with three nucleotide differences comparing with almond. Peach and nectarine sequences presented in the nucleotide position 161 a C base instead of a T, in the position 192 a G residue instead of a C and in position 209 a T instead of a C nucleotide. Apricot only presented a difference compared with almond in position 161. This difference corresponds to one of the three dissimilarities found in peach and nectarine sequences, where a C is observed instead of a T nucleotide. According to these results, apricot seems to be more closely related to almond than peach. Targeting these differences, a probe was designed in order to allow discrimination of almond from these fruits.

```
Almond EU919663         69   TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond Standard              TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond "Marcelina"           TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond "Gloriette"           TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond "Ferragnes"           TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Almond "Ferrastar"           TTTCGCTTTGCTTGCTTCTGGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA
Apricot                      ------------------------------------------------------------
Peach var. Nectarine         ------------------------------------------------------------
Peach                        TTTCGCTTTGCTTGCTTCTTGTTTTCAATGGCTGCTTAGCGGCACGCCAGTCCCAGTTGA

Almond EU919663         129  GTCCGCAGAACCAGTGCCAGCTCAACCAGCTTCAAGCCCGCGAACCCGACAACCGCATCC
Almond Standard              GTCCGCAGAACCAGTGCCAGCTCAACCAGCTTCAAGCCCGCGAACCCGACAACCGCATCC
Almond "Marcelina"           GTCCGCAGAACCAGTGCCAGCTCAACCAGCTTCAAGCCCGCGAACCCGACAACCGCATCC
Almond "Gloriette"           GTCCGCAGAACCAGTGCCAGCTCAACCAGCTTCAAGCCCGCGAACCCGACAACCGCATCC
Almond "Ferragnes"           GTCCGCAGAACCAGTGCCAGCTCAACCAGCTTCAAGCCCGCGAACCCGACAACCGCATCC
Almond "Ferrastar"           GTCCGCAGAACCAGTGCCAGCTCAACCAGCTTCAAGCCCGCGAACCCGACAACCGCATCC
Apricot                      --CCGCAGAACCAGTGCCAGCTCAACCAGCTCCAAGCCCGCGAACCCGACAACCGCATCC
Peach var. Nectarine         --CCGCAGAACCAGTGCCAGCTCAACCAGCTCCAAGCCCGCGAACCCGACAACCGCATCC
Peach                        GTCCGCAGAACCAGTGCCAGCTCAACCAGCTCCAAGCCCGCGAACCCGACAACCGCATCC

Almond EU919663         189  AGGCKGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Almond Standard              AGGCTGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Almond "Marcelina"           AGGCGGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Almond "Gloriette"           AGGCGGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Almond "Ferragnes"           AGGCGGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Almond "Ferrastar"           AGGCTGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Apricot                      AGGCGGAGGCGGGTCAGATCGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Peach var. Nectarine         AGGGGGAGGCGGGTCAGATTGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG
Peach                        AGGGGGAGGCGGGTCAGATTGAGACCTGGAACTTCAACCAGGAGGACTTCCAGTGTGCCG

Almond EU919663         249  GGGTSGCCGCCTCTCGAAT      267
Almond Standard              GGGTCGCCGCCTCTCGAAT
Almond "Marcelina"           GGGTCGCCGCCTCTCGAAT
Almond "Gloriette"           GGGTGGCCGCCTCTCGAAT
Almond "Ferragnes"           GGGTCGCCGCCTCTCGAAT
Almond "Ferrastar"           GGGTCGCCGCCTCTCGAAT
Apricot                      GGGTCGCCGCCTCTCGAAT
Peach var. Nectarine         GGGTCGCCGCCTCTCGAAN
Peach                        GGGTCGCCGCCTCTCGAAT
```

Fig. 2. Alignment of PCR products of 4 almond cultivars, almond used in reference mixtures, apricot and peach obtained by sequencing. The shadowed region of 121 bp corresponds to the PCR fragments using “outer” primers Prd6-1F/Prd6-1R.

Specificity

After DNA extraction, all samples and reference mixtures were evaluated for their amplifiability using a set of eukaryotic primers 18SEUDIR/18SEUINV (Fajardo et al., 2008), in order to prevent any resulting false-negatives. All DNA extracts tested positively with the universal primers, confirming that they contained amplifiable DNA. To assess the specificity of the designed primers for the target sequence, twelve almond cultivars, other tree nuts and several plants were tested by PCR. Specificity and cross-reactivity results for the designed primers are presented in Table 3.

All twelve almond cultivars and the almond sample used for reference mixture preparation tested positively by qualitative PCR. The similarity among almond and other fruits from the Rosaceae family was patent, since the samples apricot and peach tested positively with this set of primers when submitted to end-point PCR (data not shown), though with more fainted bands. These results evidence the homology among these fruits and consequently affect the specificity of the existing methods for almond amplification. All the other species did not amplify with the selected primers. Though the use of these primers was found to be adequate for Pru du 6 identification, some caution should be considered when analysing foods susceptible of containing peach or apricot.

Development of analytical method

Both conventional real-time PCR and single-tube nested real-time PCR systems were optimised using model standards of walnut spiked with known amounts of almond. Since no requirements for allergen testing are yet defined, the fundamentals for the assessment and comparison of the real-time PCR systems were based on the existing document of the definition of the minimum performance requirements for analytical methods of genetically modified organisms testing. The real-time PCR systems were also developed and evaluated considering the requisites defined by MIQE Guidelines (Bustin et al., 2009).

Real-time PCR system

The application of real-time PCR based on the conventional system to binary mixtures ranging from 100,000 mg/kg to 10 mg/kg (10-0.001%) enabled a relative limit of detection (LOD) of 100 mg/kg of almond in walnut (Table 4, Fig. 3a).

According to the minimum performance requirements defined by the European Network of GMO Laboratories (ENGL, 2008), the LOD should be determined considering a positive identification of the analyte at least 95% of the times, thus assuring less than 5% of false negative results. In this study, the LOD was determined assuming the lowest amount of almond DNA with positive amplification in all replicates. The limit of quantification (LOQ) achieved was equal to the LOD (100 mg/kg of almond in walnut) since the lowest amplified level was within the linear range of the calibration curve. In order to correctly evaluate a real-time PCR run, several parameters have to comply with the acceptance criteria defined for these assays.

Table 3. Results of PCR amplifications of *Prunus dulcis* clone 276NPL *prunin* gene applied to 12 almond cultivars and other plant species (26), including other tree nuts (8).

Sample name	Country of Origin	Scientific denomination	PCR
12 Almond cultivars (“Orelha de mula”, “Casa Nova”, “Pegarinhos”, “Refego”, “Marcelina”, “Verdeal”, “Gloriette”, “Duro Italiano”, “Marcona” “Ferragnes”, “Ferrastar” and “Ferradual”)	Portugal	*Prunus dulcis*	+
Almond (commercial)	Portugal	*Prunus dulcis*	+
Hazelnut	Portugal	*Corylus avellana*	-
Walnut	France	*Juglans regia*	-
Macadamia nut	Austria	*Macadamia tetraphylla*	-
Pine nut	Portugal	*Pinus pinea*	-
Brazil nut	Bolivia	*Bertholletia excelsa*	-
Pistachio	USA	*Pistacia vera*	-
Cashew	India	*Anacardium occidentale*	-
Chestnut	Portugal	*Castanea sativa*	-
Peanut	USA	*Arachis hypogaea*	-
Lupine	Portugal	*Lupinus albus*	-
Fava bean	Greece	*Vicia faba*	-
Soybean	USA	*Glycine max*	-
Maize	USA	*Zea mays*	-
Wheat	Portugal	*Triticum aestivum*	-
Rice	Portugal	*Oryza sativa*	-
Oat	Portugal	*Avena sativa*	-
Barley	Spain	*Hordeum vulgare*	-
Pumpkins seeds	Greece	*Cucurbita mixta*	-
Rapeseed	Brazil	*Brassica napus*	-
Sunflower	Brazil	*Helianthus annuus*	-
Rye	Portugal	*Secale cereale*	-
Tomato	Spain	*Solanum lycopersicum*	-
Peach	Portugal	*Prunus persica*	+
Apricot	Turkey	*Prunus armeniaca*	+
Plum	Chile	*Prunus cerasifera*	-
Cherry	Chile	*Prunus avium*	-

The parameters encompass the slope, the correlation coefficient and the PCR efficiency. A real-time PCR assay is considered of good performance when the correlation coefficient is above 0.98, the PCR efficiency ranging from 90%-110% and the slope between -3.6 and -3.1. All the assays performed with real-time PCR system presented high values of correlation and efficiency, exhibiting a correlation factor (R^2) of 0.9989, slope of -3.5032 and PCR efficiency of 93.0% (Table 4, Fig. 3c.). Thus, the referred parameters are in good agreement with the acceptance criteria defined for real-time PCR method performance (ENGL, 2008; Bustin et al., 2009). The Ct mean value established for the lowest amplified standard of walnut spiked with almond (100 mg/kg) was 37.04 cycles (Table 4).

Table 4. Real-time PCR and nested real-time PCR results for the relative detection of spiked almond in reference model mixtures.

	Real-time PCR system	Nested real-time PCR system
Spiked level (mg/kg)	Ct ± SD [a]	Ct ± SD [a]
10	nd [b]	nd
50	nd	26.89 ± 1.20 (9)
100	37.04 ± 0.70 (6)	26.18 ± 1.39 (9)
500	34.58 ± 0.68 (6)	23.18 ± 0.51 (9)
1,000	33.52 ± 0.30 (6)	21.84 ± 0.74 (9)
5,000	30.82 ± 0.20 (6)	19.58 ± 0.71 (9)
10,000	29.84 ± 0.32 (6)	18.90 ± 0.72 (9)
50,000	27.56 ± 0.16 (6)	16.35 ± 0.81 (9)
100,000	26.61 ± 0.64 (6)	15.92 ± 0.59 (9)
Correlation coefficient (R^2)	0.9989	0.9933
Slope	-3.503	-3.433
PCR efficiency (%)	93.0	95.6

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (n=6) and (n=9). [b] nd, not detected.

In order to establish the dynamic range and the absolute sensitivity of the method, almond DNA extracts 5-fold serially diluted ranging from 20 ng to 1.28 pg were evaluated using the conventional real-time PCR system developed for this study (Fig. 4a.). The set of primers and probe presented 100% PCR efficiency with approximately two additional cycles (~2 Ct) for a 5-fold dilution of almond template. Real-time PCR assays exhibited high performance, with average values of PCR efficiency of 99.5%, slope of -3.333 and R^2 of 0.9968.

The real-time PCR system allowed amplifying almond DNA until a dilution factor of 625, with a LOD of 32 pg that corresponded to 97 DNA copies (Table 5, Fig. 4a). The number of almond DNA copies was estimated according to the genome size of almond (0.33 pg) and assuming that the target sequences are single copy genes. The value for almond genome size was retrieved from the Plant DNA C-values database (RBG, Kew). This database compiles relevant information regarding several plant species such as genome size (C-value), estimation method, ploidy level, chromosome number and original references.

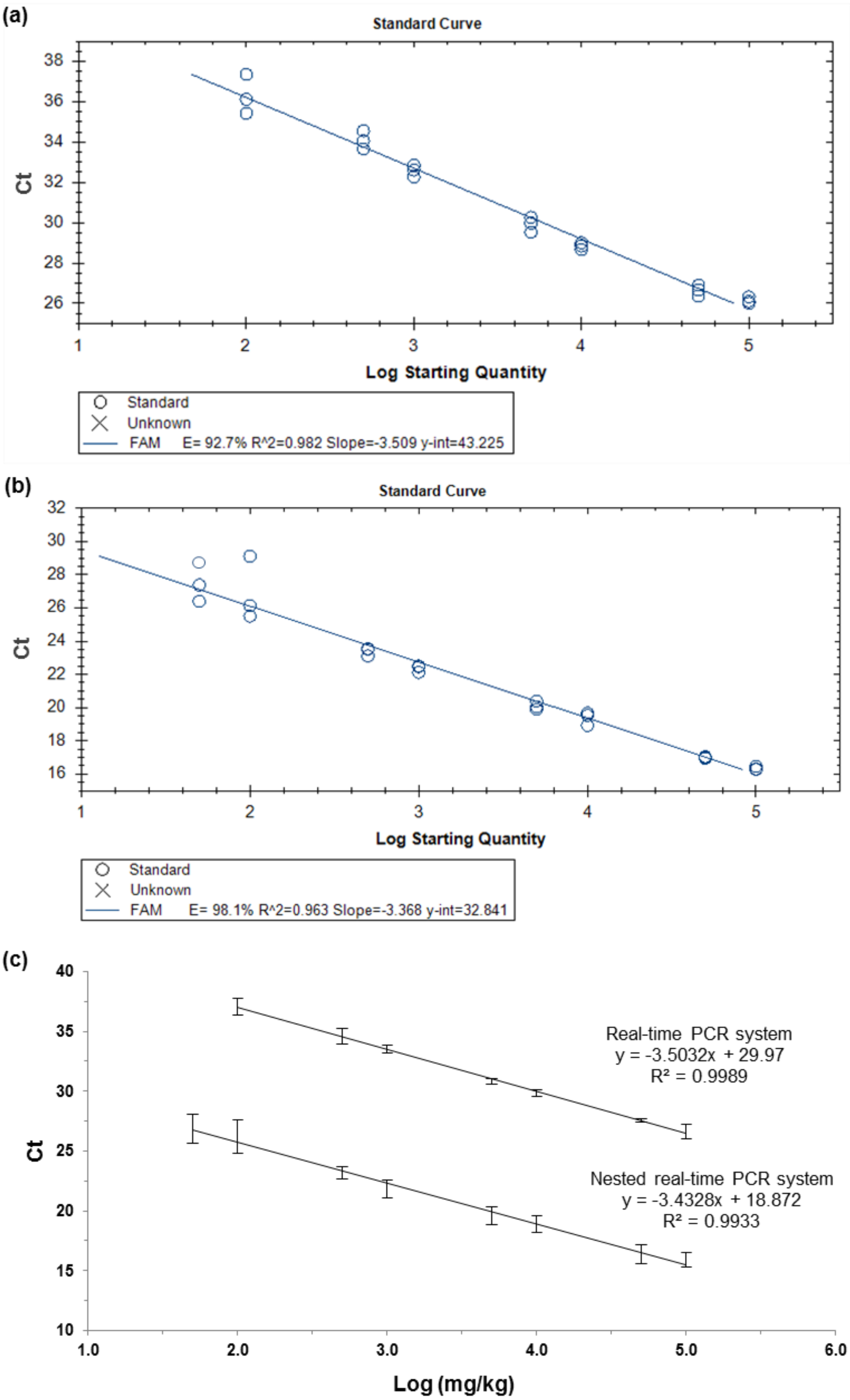


Fig. 3. Calibration curves obtained by real-time PCR (a) and nested real-time PCR (b) of reference mixtures containing 100,000; 50,000; 10,000; 5,000; 1,000; 500; 100; 50 and 10 mg/kg of almond in walnut. Average values and corresponding standard deviations of *n*=6 and *n*=9 replicates for real-time PCR and nested real-time PCR, respectively (c).

Table 5. Real-time PCR and nested real-time PCR results for the absolute detection of almond DNA.

	Real-time PCR system		Nested real-time PCR system	
Absolute quantity (pg)	Ct ± SD [a]	DNA copies [b]	Ct ± SD [a]	DNA copies [b]
1.28	nd [c]	-	24.48 ± 0.98 (9)	3.9
6.4	40.31 ± 1.65 (2)	19	23.42 ± 1.11 (9)	19
32	36.31 ± 0.90 (6)	97	20.91 ± 1.06 (9)	97
160	33.69 ± 0.23 (6)	485	18.53 ± 0.65 (9)	485
800	31.14 ± 0.36 (6)	2424	16.15 ± 0.60 (9)	2424
4,000	29.09 ± 0.28 (6)	12121	14.38 ± 0.37 (9)	12121
20,000	26.96 ± 0.26 (6)	60606	11.78 ± 0.56 (9)	60606
Correlation coefficient (R^2)	0.9968		0.9940	
Slope	-3.333		-3.114	
PCR efficiency (%)	99.5		109.5	

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (n=6) and (n=9). [b] Number of almond haploid genome copies (0.33 pg)[29]. [c] nd, not detected.

Single-tube nested real-time PCR system

The proposed single-tube nested real-time PCR system for the detection of almond allergen was optimised similarly to the conventional system using the same set of reference mixtures ranging from 100,000 mg/kg to 10 mg/kg of almond in walnut. In the case of the nested assay, the two distinct phases of amplification need to be adjusted (Table 2). The first phase was pre-established to amplify PCR fragments with 10 amplification cycles using the outer primers. Additional experiments using higher number of cycles were attempted (data not shown), selecting the value of 10 cycles in phase 1 as it presented improved relative sensitivity at best linearity.

The nested real-time PCR system achieved a relative LOD of 50 mg/kg, which was 2× lower than the value obtained with the conventional real-time PCR system (Table 4, Fig. 3b). The LOQ was the same as the LOD, considering that the lowest amplified standard was within the linear range of the calibration curve. The mean value for all the assays obtained with the new system exhibited high correlation coefficient (R^2=0.9933), appropriate slope (-3.433) and PCR efficiency (95.6%) (Table 4, Fig. 3c).

The absolute sensitivity of the nested real-time PCR system was also determined using identical almond DNA dilutions ranging from 20 ng to 1.28 pg. For these set of experiments the protocol for nested real-time PCR system was defined with 15 cycles for phase 1. The mean value for all the nested real-time PCR assays exhibited high PCR efficiency (109.5%) and correlation coefficient (R^2=0.9940) (Table 5, Fig. 4c).

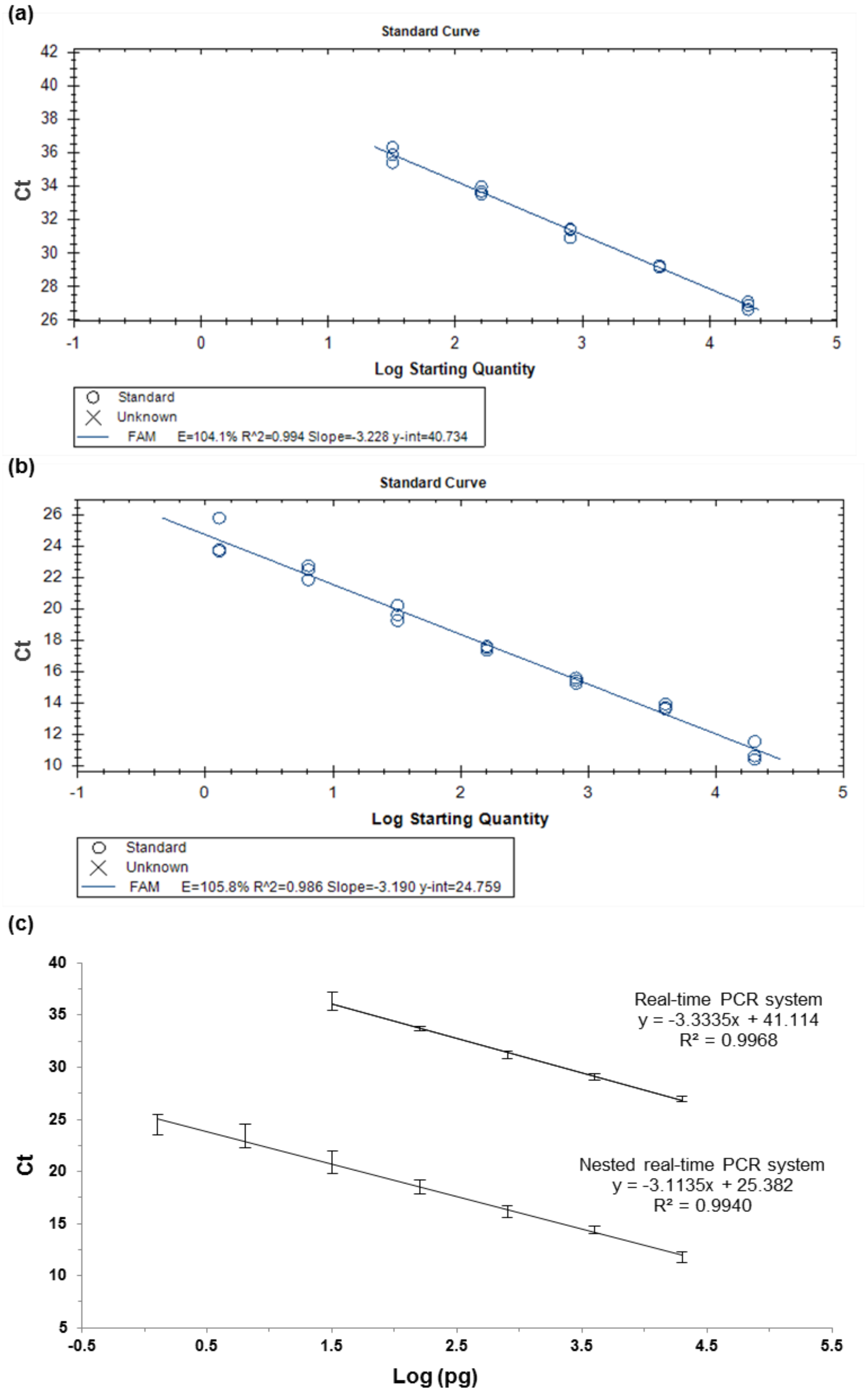


Fig. 4. Calibration curves obtained by real-time PCR (a) and nested real-time PCR (b) of almond DNA serially diluted (5-fold) from 20 ng to 1.28 pg. Average values and corresponding standard deviations of *n*=6 and *n*=9 replicates for real-time PCR and nested real-time PCR, respectively (c).

With the novel system, it was possible to increase the absolute sensitivity to 1.28 pg of almond DNA (corresponding to 3.9 DNA copies), which is 25× lower than the LOD obtained with the conventional real-time PCR system. The nested real-time PCR system allowed amplifying almond DNA until a dilution factor of 15,625. As in the previous relative quantification, the LOQ was the same as the LOD.

The nested real-time PCR system was further tested to assess any possible cross-reactivity that might occur with the closely related species of apricot and peach, leading to false positive results to almond. According to the sequencing results apricot and peach presented a very close genetic relation towards almond, with 1 and 3 nucleotide differences encountered for apricot and peach, respectively (Fig. 2). Application results of nested real-time PCR system to pure DNA extracts of almond, apricot, peach and nectarine 10-fold serially diluted, ranging from 20,000 pg to 20 pg are presented in Table 6 and Fig. 5.

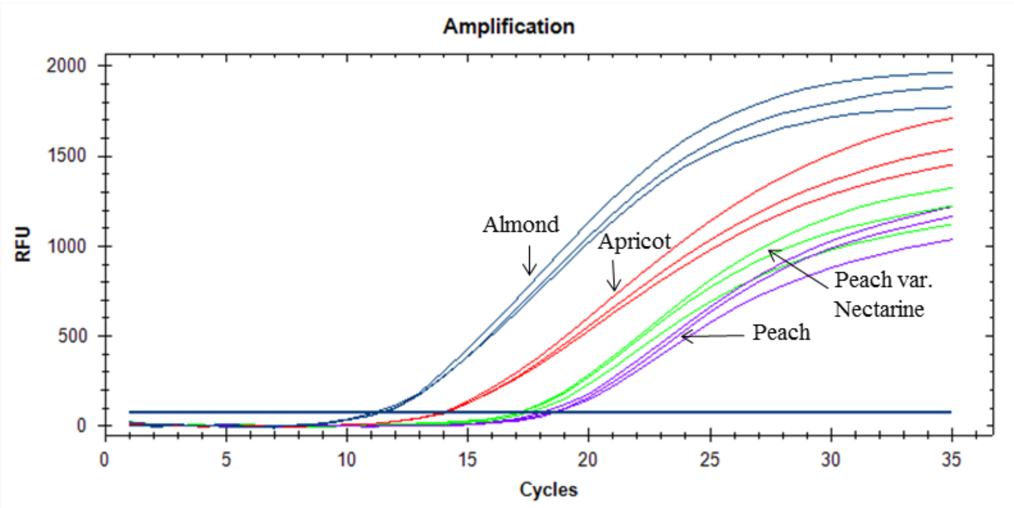


Fig. 5. Amplification curves obtained by nested real-time PCR of DNA from almond and other genetically related fruits from the Rosaceae family (apricot, peach and peach var. nectarine).

Table 6. Comparison of cycle threshold values obtained by nested real-time PCR applied to almond, apricot and peach.

	Almond	Apricot	Peach	Peach var. Nectarine
Absolute quantity (pg)	Ct ± SD [a]	Ct ± SD	Ct ± SD	Ct ± SD
20	20.57 ± 0.49 (3)	24.50 ± 0.23 (3)	nd [b]	nd
200	17.24 ± 0.27 (3)	20.47 ± 0.55 (3)	nd	22.83 ± 0.80 (2)
2,000	14.03 ± 0.20 (3)	16.99 ± 0.08 (3)	20.63 ± 1.17 (3)	19.77 ± 0.29 (3)
20,000	11.26 ± 0.18 (3)	13.99 ± 0.07 (3)	18.29 ± 0.25 (3)	17.18 ± 0.20 (3)

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (*n*=3). [b] nd, not detected.

The results evidenced a different real-time PCR profile for peach, which is in good agreement with sequencing results. Thus, for the same dilution factor for almond and peach/peach var. nectarine, amplifications presented a shift in the Ct value of approximately 8 (Table 6, Fig. 5.). Additionally, for lower amounts than 2,000 pg of peach, the nested real-time PCR system did not produce positive amplifications. Comparing almond with apricot amplification, the shift in Ct value is approximately 3, with positive amplifications until 20 pg of apricot.

DISCUSSION

The correct evaluation of the potential presence of offending ingredients using molecular methodologies is highly dependent on numerous factors, such as the type of food matrix, the allergens/DNA markers, and the chosen methodology, among others. In this work we intended to present an alternative method based on the assembly of two DNA-based methods (nested PCR and real-time PCR) for the detection of almond DNA encoding for the Pru du 6 allergen. This protein is one of the most severe allergens in almond, which highlights the importance of determining its presence, even if indirectly. Though the demonstrated relevance of Pru du 6 allergen, few studies targeted its detection in foods by DNA-based methods.

Regarding the two real-time PCR systems developed in this work, the results of the conventional real-time PCR using the “outer” primers enable the detection of 100 mg/kg of almond. This sensitivity level is in good agreement with the relative LOD for the Pru du 6 allergen in a mix matrix of 5 plant species (hazelnut, walnut, peanut, sesame and cashew) reported by Pafundo et al. (2010). By the introduction of the nested approach, it was possible to enhance the performance of the real-time PCR. Concerning the relative detection of almond, the nested real-time PCR allowed lowering 2$\times$ the LOD down to 50 mg/kg with 100% of positive replicates in all the performed assays. This LOD is comparable to the reported by Costa et al. (2012b) regarding the detection of a different almond allergen (Pru du 5). The proposed new system presents the advantage of high specificity conferred by the use of two pairs of primers and a hydrolysis probe instead of DNA intercalating dyes such as Evagreen (Costa et al., 2012b) or SYBR GreenER (Pafundo et al., 2009; 2010). Köppel et al. (2010) reported the detection of 50 mg/kg of almond, although the estimated value presented a rate of 21% of false-negatives. For quantification purposes, the estimated LOQ of 50 mg/kg establishes a supplementary improvement regarding the quantification of almond in food samples. Using the novel nested real-time PCR system, it was possible to decrease the absolute level of detection to 1.28 pg of almond representing 3.9 DNA copies. Pafundo et al. (2009) reported an apparently lower level of almond detection (1 DNA copy) on basis of an almond genome size of 0.30 ng, which is approximately 1,000 times higher than the 0.33 pg, reported by Baird, Estager, and Wells (1994) and the Plant DNA C-values database (RGB, Kew). Thus, taking this fact into consideration, the proposed method is able to detect a much inferior level of almond than the reported value of Pafundo et al. (2009).

Besides tracing small amounts of Pru du 6 allergen, the single-tube nested real-time PCR presents high performance criteria and apparent robustness. The developed approach was not affected by shifts in temperature, time and cycle number, considering the existence of two different reaction protocols within the same assay. In both real-time PCR systems, the tested parameters complied with the criteria established by the ENGL (2008) and with the MIQE guidelines (Bustin et al. 2009), thus evidencing their adequacy for the detection of almond Pru du 6 allergen.

Comparing the performances of the two real-time PCR methods, the single-tube nested real-time PCR proved to be a specific and sensitive technique for the detection of almond in foods. Some caution should be taken when analysing samples susceptible of containing apricot or peach since the system can also amplify these fruits, but only if the quantity of initial DNA is high. The homology between almond and apricot regarding DNA encoding the Pru du 6 allergen is high. Conversely, the existence of a single difference in the DNA sequences of almond and apricot is enough to produce a shift in the Ct value of approximately 3 for the same DNA concentration. In identical conditions, almond and peach presented a shift of 8 Ct, which is a consequence of the 3 nucleotide mismatches encountered in respective sequences. Almond and other fruits from the Rosaceae family have been extensively described as genetically related with high homology, still the molecular methods available so far present very few (Köppel et al., 2010) or no information at all (Pafundo et al., 2009; 2010) about possible cross-reactivity among them.

In summary, the developed single-tube nested real-time PCR method presented in this work constitutes an alternative approach to detect almond at trace levels in foods. The effective application of the single-tube nested real-time PCR to different DNA targets like almond, hazelnut (Costa et al., 2012c) or peanut (Bergerová et al., 2011) highlights the usefulness of this new tool and its potentiality for the identification of diverse allergens in foods, for which further research work is still required.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011, by the project "Improvement of molecular biology methods for authenticity assessment and detection of allergens in foods" (FCT/2489/3/6/2011/S) of Technological and Scientific Cooperation FCT/Slovakia and by University of Porto "Projectos Pluridisciplinares" IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

Alasalvar, C., & Shahidi, F. (2008). Tree nuts: composition, phytochemicals, and health effects: an overview. In C. Alasalvar, & F. Shahidi, (Eds.), *Tree Nuts: Composition, Phytochemicals, and Health Effects* (pp 1-6). Boca Raton: CRC Press.

Albillos, S. M., Menhart, N., & Fu, T.-J. (2009). Structural stability of amandin, a major allergen from almond (*Prunus dulcis*), and its acidic and basic polypeptides. *Journal of Agricultural and Food Chemistry*, *57*, 4698-4705.

Baird, W. V., Estager, A. S., & Wells, J. K. (1994). Estimating nuclear DNA content in Peach and related diploid species using laser flow cytometry and DNA hybridization. *Journal of the American Society for Horticultural Science*, *119*, 1312-1316.

Bergerová, E., Brežná, B., & Kuchta, T. (2011). A novel method with improved sensitivity for the detection of peanuts based upon single-tube nested real-time polymerase chain reaction. *European Food Research and Technology*, *232*, 1087-1091.

Bignardi, C., Elviri, L., Penna, A., Careri, M., & Mangia, A. (2010). Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *Journal of Chromatography A*, *1217*, 7579-7585.

Burney, P., Summers, C., Chinn, S., Hooper, R., Van Ree, R., & Lidholm, J. (2010). Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy*, *65*, 1182-1188.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments *Clinical Chemistry*, *55*, 611-622.

Costa, J., Mafra, I., Carrapatoso, I., & Oliveira, M. B. P. P. (2012a). Almond allergens: molecular characterization, detection, and clinical relevance. *Journal of Agricultural and Food Chemistry*, *60*, 1337-1349.

Costa, J., Mafra, I., & Oliveira, M. B. P. P. (2012b). High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. *Food Chemistry*, *133*, 1062-1069.

Costa, J., Mafra, I., Kuchta, T., & Oliveira, M. B. P. P. (2012c). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry*, *60*, 8103-8110.

ENGL (2008). European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing, European Commission: Brussels. Available online at: http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm (Website last accessed on March 19, 2012).

Fajardo, V., Gonzalez, I., Martin, I., Rojas, M., Hernandez, P. E., Garcia, T., et al. (2008). Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. *Meat Science*, *79*, 289-298.

FDA (2003). Food and Drug Administration. Administration qualified health claims: letter of enforcement discretion-nuts and coronary heart disease, Docket No. 02P-0505; Washington DC.

Garber, E., & Perry, J. (2010). Detection of hazelnuts and almonds using commercial ELISA test kits. *Analytical and Bioanalytical Chemistry*, *396*, 1939-1945.

Heick, J., Fischer, M., & Pöpping, B. (2011). First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *Journal of Chromatography A*, *1218*, 938-943.

Holck, A., Diaz-Amigo, C., Kerbach, S., Popping, B., Mustorp, S., & Axelsson, C. E. (2011). Detection of allergens in food. In M. B. P. P. Oliveira, I. Mafra, & J. S. Amaral, (Eds.), *Current topics on food authentication* (pp. 173-210), Kerala: Transworld Research Network.

Jin, T., Albillos, S. M., Guo, F., Howard, A., Fu, T.-J., Kothary, M. H., et al. (2009). Crystal structure of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. *Journal of Agricultural and Food Chemistry*, 57, 8643-8651.

Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., & Waiblinger, H.-U. (2010). Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *European Food Research and Technology*, *230*, 367-374.

Pafundo, S., Gullì, M., & Marmiroli, N. (2009). SYBR® GreenER™ Real-Time PCR to detect almond in traces in processed food. *Food Chemistry*, *116*, 811-815.

Pafundo, S., Gullì, M., & Marmiroli, N. (2010). Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. *Analytical and Bioanalytical Chemistry*, *396*, 1831-1839.

RBG (Royal Botanic Gardens) Kew, Plant DNA C-values database, Surrey, Canada. Available online at: http://data.kew.org/cvalues/ (Website last accessed: August 8, 2012).

Rejeb, S. B., Abbott, M., Davies, D., Cléroux, C., & Delahaut, P. (2005). Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Additives and Contaminants*, *22*, 709-715.

Röder, M., Vieths, S., & Holzhauser, T. (2011). Sensitive and specific detection of potentially allergenic almond (*Prunus dulcis*) in complex food matrices by TaqMan® real-time polymerase chain reaction in comparison to commercially available protein-based enzyme-linked immunosorbent assay. *Analytica Chimica Acta*, *685*, 74-83.

Roux, K. H., Teuber, S. S., & Sathe, S. K. (2003). Tree nut allergens. *International Archives of Allergy and Immunology*, *131*, 234-244.

Sathe, S. K., Wolf, W. J., Roux, K. H., Teuber, S. S., Venkatachalam, M., & Sze-Tao, K. W. C. (2002). Biochemical characterization of amandin, the major storage protein in almond (*Prunus dulcis* L.). *Journal of Agricultural and Food Chemistry*, *50*, 4333-4341.

Scheibe, B., Weiss, W., Ruëff, F., Przybilla, B., & Görg, A. (2001). Detection of trace amounts of hidden allergens: hazelnut and almond proteins in chocolate. *Journal of Chromatography B: Biomedical Sciences and Applications*, *756*, 229-237.

Sicherer, S. H., Muñoz-Furlong, A., Godbold, J. H., & Sampson, H. A. (2010). US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *Journal of Allergy and Clinical Immunology*, *125*, 1322-1326.

Sicherer, S. H., & Sampson, H. A. (2006). Food allergy. *Journal of Allergy and Clinical Immunology*, *117* (2, Supplement 2), S470-S475.

Wang, W., Li, Y., Zhao, F., Chen, Y., & Ge, Y. (2011). Optical thin-film biochips for multiplex detection of eight allergens in food. *Food Research International*, *44*, 3229-3234.

van Hengel, (2007). A. J. Declaration of allergens on the label of food products purchased on the European market. *Trends in Food Science and Technology, 18*, 96-100.

(Submitted)

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols

Joana Costa, Vítor S. Melo, Cristina G. Santos, M. Beatriz P. P. Oliveira, Isabel Mafra*

REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal

*Corresponding author. Tel.: +351 220428640; fax: +351 226093390. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

Chocolates are often restricted to allergic individuals due to its precautionary labelling regarding the presence of potentially allergenic ingredients, such as tree nuts. Reliable labelling of allergenic ingredients is of major importance for susceptible individuals, requiring appropriate detection techniques. The aim of the present work was to compare different DNA extraction methods from chocolate matrices for the effective application of molecular techniques to tree nut allergen detection. For this study, DNA from almond or hazelnut model chocolates was extracted using seven selected protocols: CTAB-PVP, Wizard with and without RNase, Wizard-PVP with and without RNase, Wizard Magnetic and Nucleospin. The extracts were assessed for their suitability for amplification by qualitative PCR and real-time PCR. From the evaluated protocols, Nucleospin presented the best results for almond and hazelnut amplification, reaching a limit of detection of 0.005% with high PCR efficiency, linearity and range of amplification. These results highlight the importance of the DNA extraction protocol in the particular case of food allergens from complex matrices such as chocolate, from which sensitivity is a key parameter.

Keywords Food allergens; almond; hazelnut; chocolate; DNA extraction; real-time PCR.

INTRODUCTION

The prevalence of food allergies has been increasing, especially in industrialised countries, being estimated to affect up to 3-4% of adult population and 6-8% of young children (Sicherer & Sampson, 2006). Among food allergies, abnormal immunological responses to tree nut consumption are a frequent cause of severe allergic reactions in sensitised individuals (Bettazzi et al., 2008). Almonds (*Prunus dulcis*) and hazelnuts (*Corylus avellana*), as part of the tree nut group, are responsible for triggering mild to life-threatening reactions in allergic individuals (Sampson, 2003). Since the avoidance of food allergens is the only effective means of protecting the health of *sensitised* individuals, EU legislation has issued the mandatory labelling of allergenic food ingredients. Regulation (EU) 1169/2011 provides a list of 14 groups of certain substances or products causing allergies or intolerances that are required to be emphasised from the rest of the list of ingredients, regardless of their quantity. However, the total avoidance of food allergens is difficult to accomplish, since processed foods may contain natural allergens added either deliberately as food ingredients or unintentionally as the result of cross-contaminations during production, shipping or storage (Mustorp, Dromtorp, & Holck, 2011; Poms, Klein, & Anklam, 2004). To comply with the mandatory legislation, the food industry often declares "may contain traces of tree nuts", as precautionary labelling that severely restricts the free choice of several processed foods from the point of view of the allergic individuals.

Almonds and hazelnuts are used in a wide variety of foodstuffs such as chocolates, which are very popular among individuals of all ages, especially children. Due to the lack of legal thresholds for the presence of food allergens and to comply with the mandatory legislation, chocolates regularly specify precautionary labelling. Consequently, proper analytical methodology is required to verify the adequacy of allergen label statements and to evaluate the potential risk to food-sensitive consumers (Krska et al., 2011). Presently, several immunochemical techniques, namely commercial ELISA and lateral flow device kits can be used for the detection of almond and hazelnut protein residues in chocolates (Akkerdaas et al., 2004; Holzhauser & Vieths, 1999; Koppelman et al., 1999; Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005). DNA-based methods have also become valuable tools for the detection of minute amounts of almond and hazelnut in a wide range of food products (Bettazzi et al., 2008; Costa, Mafra, & Oliveira, 2012a; Costa, Mafra, Kuchta, & Oliveira, 2012b; D'Andrea, Coisson, Travaglia, Garino, & Arlorio, 2009; Holzhauser, Wangorsch, & Vieths, 2000; Köppel et al., 2010; Pafundo, Gullì, & Marmiroli, 2010). In highly processed foods, polymerase chain reaction (PCR) techniques have been proving to be useful alternatives owing to the higher stability of DNA molecules compared with proteins, avoiding the problems of cross-reactivity. More recently, real-time PCR has been preferred to qualitative PCR owing to its increased sensitivity and specificity in detecting minute amounts of allergens in foods, whose application has been reported in a wide range of food products. Though several reports include real-time PCR detection of almond and hazelnut in foods, the number of applications to chocolate matrix and the range of analysed samples are limited, without estimating any limits of detection (Arlorio, Cereti, Coisson, Travaglia, & Martelli, 2007; Costa et al., 2012a; Costa et al., 2012b; D'Andrea et al., 2009; Köppel et al., 2010; Pafundo et al., 2010; Piknová, Pangallo, & Kuchta, 2008; Schöringhumer, Redl, & Cichna-Markl, 2009). Only very recently, Röder, Vieths and Holzhauser (2011) demonstrated to achieve a real-time PCR sensitive detection of almond in spiked chocolate. Thus, to effectively apply real-time PCR to detect tree nut allergens in chocolate, it is important to investigate and to assess the limits of detection (LOD) of almond and hazelnut in this kind of food matrix. However, highly processed and complex food matrices such as chocolate are very rich in substances like polyphenols, carbohydrates and aromatic compounds that may interfere and inhibit the DNA amplification. Consequently, the method used to extract DNA from chocolate matrices should be critically chosen and/or optimised to ensure efficient recovery of the nucleic acid and the removal of potential PCR inhibitors.

The aim of this study was to compare the performance of seven DNA extraction protocols to enable the effective application of real-time PCR for the detection of trace amounts of almond and hazelnut in chocolates. To achieve this goal, chocolate model mixtures were prepared in the laboratory by spiking known amounts of almond or hazelnut until trace levels, which were used to determine the respective LOD and limit of quantification (LOQ) in chocolates. DNA extraction protocols, including commercial kits and in-house prepared protocols were optimised and compared for their suitability for DNA amplification by qualitative PCR and real-time PCR with the use of specific fluorescent hydrolysis probes for almond and hazelnut detection.

MATERIAL AND METHODS

Reagents and equipment

For the preparation of lysis buffers and other solutions used in CTAB and Wizard methods, reagents of molecular biology grade purchased from Sigma-Aldrich (St. Louis, MO, USA) included: cetyltrimethylammonium bromide (CTAB), trizma base, ethylenediaminetetraacetic acid (EDTA), polyvinylpyrrolidone (PVP-40), β-mercaptoethanol and guanidine hydrochloride. Ethanol, isopropanol, sodium chloride and dodecyl sulphate sodium salt of analytical grade were acquired from Merck (Darmstadt, Germany) and chloroform from Fluka (Madrid, Spain). GelRed (nucleic acid gel stain) for agarose gel staining was obtained from Biotium (Hayward, CA, USA), SGTB buffer 20× for electrophoresis from GRISP Research Solutions (Porto, Portugal), RNase A from AppliChem (Darmstadt, Germany) and proteinase K from Bioron (Ludwigshafen, Germany). The commercial kits used in this study comprised the Nucleospin® food kit acquired from Macherey-Nagel (Düren, Germany) and the Wizard® Magnetic DNA purification system for food from Promega (Madison, WI, USA). The columns and DNA purification resin used in Wizard-based methods were obtained in Wizard® Plus MiniPreps DNA purification system from Promega (Madison, WI, USA). For qualitative PCR, the dNTP were bought from Invitrogen (Carlsbad, CA, USA) and the enzyme SuperHot Taq-DNA-Polymerase from Genaxxon Bioscience (Ulm, Germany). For real-time PCR, the mix SsoFast™ Probes Supermix was acquired from Bio-Rad (Hercules, CA, USA).

For sample preparation, a laboratory knife mill (Grindomix GM200) from Retsch (Haan, Germany) was used. For simultaneous stirring and temperature control during DNA extraction, a thermal block (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany) was employed. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital Science™ DC120 (Rochester, NY, USA). UV spectrometric DNA quantification was carried out on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA). Qualitative PCR assays were performed on a thermal cycler MJ Mini from Bio-Rad Laboratories and the real-time PCR assays on a fluorometric thermal cycler CFX96 Real-time PCR Detection System with Bio-Rad CFX Manager 3.0 software from Bio-Rad Laboratories (Hercules, CA, USA).

Spiking chocolates

Almond and hazelnut kernels were grounded and homogenised separately in the laboratory knife mill using different material and containers previously treated with DNA decontamination solution. A total of 11 model mixtures containing 10% down to 0.0001% of almond or hazelnut were prepared using chocolate with 41% of cocoa formerly tested for the presence of unintended almond and hazelnut residues. Chocolates spiked with almonds or hazelnuts were prepared in different days to prevent any possible unintentional cross-contamination. To prepare model mixtures, the chocolate was melted and maintained at 40 ºC during the entire procedure to ensure correct and complete homogeneity of the materials. The first mixture containing 10% of almond or hazelnut was prepared by adding 20 g of almond or hazelnut to 180 g of melted chocolate. All the following binary mixtures were prepared by serial addition of melted chocolate. The solidified spiked chocolates were further chopped into pieces of approximately 0.3 mm of diameter (Grindomix GM200) and immediately stored at -20 ºC until DNA extraction.

DNA extraction methods

Chocolate model mixtures were extracted using seven different protocols with several modifications: CTAB-PVP, Wizard, Wizard with RNase, Wizard-PVP, Wizard-PVP with RNase, Wizard Magnetic and Nucleospin methods. The first five were based on in-house developed protocols as described by Mafra, Silva, Moreira, da Silva, & Oliveira (2008) with some modifications. The latter two protocols comprised the use of the commercial kits: Wizard® Magnetic DNA purification system for food and Nucleospin® food kit.

CTAB-PVP method

The chocolate samples (200 mg) were transferred to a 2 mL sterile reaction tube followed by the addition of 1,000 µL of CTAB extraction buffer (2% of CTAB (w/v), 0.1 M of Tris, 1.4 M of NaCl, 1% of PVP-40 (w/v), 0.02 M of EDTA, pH 8.0) pre-heated at 65 ºC and 20 µL of β-mercaptoethanol. After incubating at 65 ºC for 1 h in a thermal block with continuous stirring, the mixture was centrifuged (15 min, 18500×*g* at 4 ºC) and 700 µL were collected and centrifuged again for 5 min, in the same conditions. About 600 µL of collected supernatant were extracted with 500 µL of chloroform and centrifuged (10 min, 12,000×*g* at 4 ºC). The aqueous phase was transferred to a new reaction tube, mixed with the double volume of CTAB precipitation solution (0.5% of CTAB (w/v), 0.04 M of NaCl) and incubated for 1 h at room temperature. After sample centrifugation (10 min, 12,000×*g* at 4 ºC), the supernatant was rejected, the precipitate was dissolved in 350 µL of 1.2 M of NaCl and extracted with 350 µL of chloroform, followed by a new centrifugation in the same conditions. The upper phase was then collected and mixed with 0.6 volume parts of isopropanol (80%, v/v) at -20 ºC. The mixture was centrifuged (10 min, 12,000×*g* at 4 ºC) and the supernatant discarded. The pellet was washed in 500 µL of ethanol solution (70%, v/v) at -20 ºC. After centrifugation, the supernatant was carefully rejected by pipetting, the pellet was dried for 30 min at 50 ºC and the DNA was dissolved in 100 µL of Tris–EDTA buffer (1 mM of Tris, 0.1 mM of EDTA).

Wizard method

To each 200 mg of grounded chocolate, 860 µL of TNE extraction buffer (10 mM of Tris, 150 mM of NaCl, 2 mM of EDTA, 1% of SDS), 100 µL of 5 M of guanidine hydrochloride solution and 40 µL of proteinase K solution (20 mg mL^{-1}) were added. After incubating at 60 ºC for 3 h in a thermal block with continuous stirring, the mixture was centrifuged (15 min, 18,500×*g* at 4 ºC) and 700 µL were collected and centrifuged again for 5 min using the same conditions. A volume of about 600 µL of supernatant was collected and mixed with 1 mL of Wizard® DNA purification resin. The mixture was pushed through a Wizard column mounted with a 2 mL syringe. The DNA–resin mix was washed twice with 2 mL isopropanol solution (80%, v/v). After the washing steps, each column was centrifuged for 2 min at 10,000×*g*, dried for 5 min at room temperature and mounted on a new reaction tube. The DNA was eluted by the addition of 100 µL of Tris-EDTA buffer at 70 ºC, incubation for 1 min and centrifugation (1 min, 10,000×*g*).

Wizard with RNase method

This method was performed as described for Wizard method (section 2.3.2), but including the addition of RNase after the lysis step. Next to the second centrifugation and prior to the addition of Wizard® DNA purification resin, 4 µL of RNase A (10 mg mL^{-1}) were added to the supernatant and the mixture was incubated for 10 min at 37 ºC with smooth stirring.

Wizard-PVP method

This method was performed as described for Wizard method (section 2.3.2), but using a modified TNE extraction buffer with PVP (10 mM of Tris, 150 mM of NaCl, 2 mM of EDTA, 1% of SDS, 1% of PVP-40 (w/v)), followed by the addition of 20 µL of β-mercaptoethanol to the mixture.

Wizard-PVP with RNase method

This method was carried out as described for Wizard method (section 2.3.2), but including both modifications described in sections 2.3.3 and 2.3.4.

Wizard Magnetic method

The Wizard Magnetic protocol was done according to the suggestions of the manufacturer with slight modifications. Each chocolate sample (200 mg) was transferred to a 2 mL sterile reaction tube followed by the addition of 500 µL of Buffer A and 5 µL of RNase A (4 mg mL^{-1}). After vigorous stirring, 250 µL of Buffer B were mixed, followed by incubation at room temperature for 10 min with occasional stirring and by the addition of 750 µL of precipitation solution. The mixture was centrifuged (18,500×*g*, for 10 min at 4 ºC) and 1 mL of supernatant was collected to a new sterile reaction tube, blended with 40 µL of MagneSil® paramagnetic particles and with 0.8 volume parts of isopropanol. The mixture was then incubated for 5 min at room temperature with occasional mixing by inversion. The tube was placed in a PolyATtract® system 1000 magnetic separation stand for about 1 min, until all the particles were attracted to the support. The clear solution was carefully rejected, and the particles were washed twice with 500 µL and 250 µL of buffer B solution, respectively. The particles were further washed twice with 1 mL of ethanol 70% (v/v) and dried for 30 min at 50 ºC. The DNA was released from the magnetic particles by adding 100 µL of Tris-EDTA buffer at 65 ºC, incubation for 5 min with soft stirring, centrifugation (1 min, 13,000×*g*) and the supernatant transferred to a new sterile reaction tube.

Nucleospin method

The Nucleospin method was performed according to the manufacturer's instructions with some modifications. To each 200 mg of chocolate sample, 700 µL of lysis solution CF pre-heated at 65 ºC and 10 µL of proteinase K (20 mg mL^{-1}) were added. After 1 h incubation at 65 ºC with continuous stirring, 4 µL of RNase A (10 mg mL^{-1}) were mixed and incubated for 10 min at 37 ºC with soft stirring. Subsequently, the mixtures were centrifuged for 10 min (18,500×*g* at 4 ºC) and the supernatant (550 µL) transferred to a new sterile reaction tube. A second centrifugation step was performed for 5 min in the same conditions and 450 µL of supernatant were collected to a new sterile reaction tube, to which equal volumes of precipitation solution C4 and ethanol 100% were added to each sample. The mixture was homogenised by inversion and eluted through the spin column by centrifugation (1 min, 13,000×*g*). The column was then washed twice with 400 µL of CQW solution and twice (700 µL and 200 µL) with C5 solution, with 1 min centrifugations (13,000×*g*) between washings and a 2 min final centrifugation. The DNA was eluted from the column by the addition of 100 µL of CE solution at 70 ºC, incubation at room temperature for 5 min and centrifugation (1 min, 13,000×*g*).

The extractions were performed at least twice for each spiking level and method, keeping the extracts at -20 ºC until further analysis.

Target gene selection

The detection of almond was performed using primers and probe retrieved from the literature (Costa, Oliveira, & Mafra, 2013) targeting the *prunin* gene, which partially encodes the allergen amandin. For hazelnut detection, specific primers and probe targeting the *hsp1* gene that encodes a low molecular weight heat-shock protein were also selected from the literature (Piknová et al., 2008).

The oligonucleotide primers and probes are listed in Table 1 and were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Table 1. Key data of primers and probes designed to target *prunin* and *hsp1* genes (Genbank accession no. EU919663 and AF021807) for almond and hazelnut detection, respectively.

Oligonucleotides	Sequence (5'-3')	Amplicon (bp)	References
Almond			
Prd6-1F	CCGCAGAACCAGTGCCAGCT		
Prd6-1R	CCCCGGCACACTGGAAGTCCT	121	Costa et al. (2013)
Prd6-1P	FAM-GCTTCAAGCCCGCGAACCCGACAAC-BHQ2		
Hazelnut			
Nocc2F	GGCAAGTTCGTGAGCAGGTTC		
Nocc1Rbis	CTTTCGGAATAGTCACAGTGAG	100	Piknová et al. (2008)
Nocc1P	FAM-CCTGACGATGCGATGCTCGACCAG-BHQ2		

Qualitative PCR

Qualitative PCR amplification was carried out in 25 µL of total reaction volume containing 2 µL of DNA extract of almond or hazelnut model chocolates (20 ng), 670 mM of Tris–HCl (pH 8.8), 160 mM of $(NH_4)_2SO_4$, 0.1% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq-DNA Polymerase, 3.5 or 3.25 mM of $MgCl_2$ and 200 nM of each primer Prd6-1F/Prd6-1R or 160 nM of each primer Nocc2F/Nocc1Rbis, for almond or hazelnut, respectively (Table 1). The reactions were performed in a thermal cycler using the following programme: initial denaturation at 95 ºC for 5 min; 40 cycles at 95 ºC for 30 s, 64 ºC or 65 ºC for 30 s, using almond or hazelnut primers, respectively, and 72 ºC for 30 s; with a final extension at 72 ºC for 5 min.

The amplified fragments were analysed by electrophoresis in 1.5% agarose gel containing 1x GelRed for staining and performed in 1x SGTB buffer for 20-25 min at 200 V. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital Science™ DC120. Each extract was amplified at least in duplicate assays.

Real-time PCR

Amplifications by real-time PCR were performed in 20 µL of total reaction volume containing 2 µL of DNA extract of almond or hazelnut chocolate standards (20 ng), 1× of SsoFast™ Probes Supermix, 300 nM of each primer and 150 nM of probe for both almond and hazelnut targets (Table 1). The assays were carried out in a fluorometric thermal cycler using the following conditions: 95 ºC for 5 min; 50 cycles at 95 ºC for 15 s and 65 ºC for 45 s, with fluorescence signal collection at the end of each cycle. Data was processed using the software Bio-Rad CFX Manager 3.0. Each extract was amplified in triplicate or quadruplicate in two independent assays.

Statistical analysis

A one-way ANOVA with the statistical programme IBM SPSS STATISTICS (20.0 package, IBM Corporation, New York, USA) was performed to evaluate the differences among DNA yield and Ct values obtained with seven extraction methods. All data were previously assessed for normality and homogeneity of variances by Shapiro-Wilk and Leven's tests, respectively. When these tests exhibited significance, individual means were compared using Tukey's test. Significant differences were considered when $p < 0.05$.

RESULTS

In the present work, seven DNA extraction protocols, namely, CTAB-PVP, Wizard with and without RNase, Wizard-PVP with and without RNase, Wizard Magnetic and Nucleospin were tested for their adequacy to obtain amplifiable DNA from different chocolate matrices. The use of RNase A enzyme aimed at removing RNA residues to provide purer and more stable DNA extracts. Following the manufacturer's instructions, RNase A was always used with Nucleospin and Wizard Magnetic methods. For CTAB-PVP method, the addition of RNase A was also attempted. Nevertheless, the resultant DNA yield was excessively low, which restricted its addition.

Another important issue evaluated in this study concerned the amount of template DNA used for PCR amplifications. Accordingly, different DNA concentrations were tested, namely 50, 25, 10 and 5 ng μL^{-1} (data not shown), with the best results for almond and hazelnut amplification in chocolate matrices obtained for 10 ng μL^{-1} extracts. Higher or lower DNA concentrations led to the production of faint bands or even failed to amplify the target fragments. The presence of PCR inhibitors might justify the difficulties of amplifying higher concentrations, therefore 20 ng of template DNA were used in all tested protocols.

Almond

Analysis of the extracted DNA

The estimated results for DNA concentration and purity of almond chocolate extracts using different methods of DNA extraction are listed in Table 2. The extracts with lowest DNA content were obtained with the CTAB-PVP method, followed by the Wizard Magnetic and the Wizard methods, with average concentrations of 42.8, 48.0 and 52.2 ng μL^{-1}, respectively. Despite the low DNA yields, these extracts exhibited high level of purity with A_{260}/A_{280} ratio above 1.63. The extracts obtained with the Wizard-PVP protocols with and without the addition of RNase revealed relatively high DNA contents, 84.3 and 93.1 ng μL^{-1}, respectively, but low purities (1.2). Nucleospin method produced extracts with the significantly highest DNA concentration (117.5 ng µL^{-1}), although with purity below the ideal (A_{260}/A_{280}=1.41). All DNA extracts from different protocols were tested for their suitability for amplification targeting the *prunin* gene using qualitative and real-time PCR techniques.

Table 2. DNA concentration and purity of the extracts of chocolate model mixtures spiked with almond or hazelnut obtained with different methods

Extraction Method	Chocolates spiked with almond		Chocolates spiked with hazelnut	
	DNA* ng µL^{-1}	Purity** A_{260}/A_{280}	DNA* ng µL^{-1}	Purity** A_{260}/A_{280}
Wizard-PVP	93.1 ± 15.1ab	1.24 ± 0.12b	98.1 ± 5.1a	1.31 ± 0.15b
Wizard-PVP with RNase	84.3 ± 13.7bc	1.23 ± 0.10b	82.4 ± 10.4a	1.36 ± 0.23b
Wizard	52.2 ± 12.0cd	1.41 ± 0.27ab	47.8 ± 14.5b	1.63 ± 0.30ab
Wizard with RNase	75.4 ± 15.0bc	1.77 ± 0.07ab	50.2 ± 2.4b	1.96 ± 0.22a
Wizard Magnetic	48.0 ± 18.4cd	1.63 ± 0.41ab	77.0 ± 14.4a	1.40 ± 0.22b
CTAB-PVP	42.8 ± 13.2d	1.87 ± 0.59a	38.4 ± 8.5b	1.52 ± 0.12b
Nucleospin	117.5 ± 17.5a	1.41 ± 0.06ab	97.6 ± 15.4a	1.34 ± 0.12b

*Values represent mean (*n*=10) and standard deviation (SD). **$A_{260,}$ absorbance at 260 nm; A_{280}, absorbance at 280 nm. In each column, different letters indicate significant differences among values of DNA concentration or purity ($p < 0.05$).

Qualitative PCR detection

The application of end-point PCR to model chocolates spiked with almond allowed the amplification of *prunin* gene fragments of 121 bp. Fig. 1 presents the PCR results of model chocolates extracted with different methods. The DNA isolated with Wizard-PVP method only revealed positive amplifications for chocolate mixtures containing 10% and 1% of almond. The addition of RNase to the previous protocol did not improve PCR results. The presence of PCR inhibitors in the extracts obtained with these protocols could be a probable cause for the low amplification level since they produced significantly lower DNA purity.

Wizard method enabled amplifying strong PCR fragments for 10% and 1%, but only faint bands for 0.1% and 0.01%. By adding RNase to the Wizard protocol, it was possible to intensify the bands of the 3 highest concentrations (10%, 1% and 0.1% of almond), but still no products for the lowest levels. The Wizard Magnetic method seemed to increase sensitivity down to 0.05%, though no clear band gradient could be observed. Regarding the CTAB-PVP method, in spite of producing the significantly lowest DNA yields (Table 2), all the mixtures amplified the expected fragment, with intermediate concentrations of 0.1% and 0.5% exhibiting faint bands. The final tested Nucleospin method allowed amplifying all the levels of spiked almond, revealing a clear gradient with decreasing almond percentage. From the assessed protocols to isolate amplifiable almond DNA from model chocolates, the Nucleospin method presented the best amplification results and DNA yield.

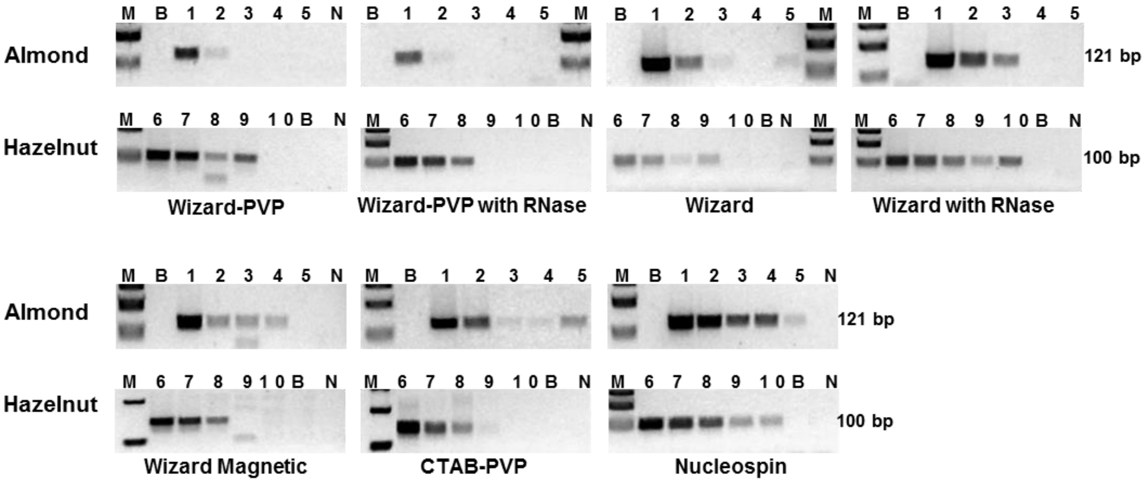


Fig. 1. Agarose gel electrophoresis of PCR products using primers Prd6-1F/Prd6-1R or Nocc2F/Nocc1Rbis targeting prunin or hsp1 genes, respectively, of model chocolates and extracted with different methods: lanes 1 to 5, model chocolates spiked with 10%, 1%, 0.1%, 0.05% and 0.01%, of almond or hazelnut; N, negative control; B, extraction blank; M, 100 bp ladder (Bioron, Ludwigshafen, Germany), M', 50 bp FastRuler™ Low Range DNA ladder (Fermentas GMBH, St. Leon-Rot, Germany).

Real-time PCR detection

In the case of almond in chocolate matrix, DNA extracts obtained with Wizard-PVP and Wizard-PVP with RNase methods (Fig. 1) were not further analysed by real-time PCR, considering the poor performance of qualitative PCR. Regarding the other five protocols, real-time PCR amplification using hydrolysis fluorescent probe was carried out to assess their performance for quantitative analysis (Table 3).

DNA isolated with Wizard method presented positive amplification until a sensitivity of 0.01% for almond with adequate PCR efficiency (112.9%) and linear correlation (R^2=0.956) (Table 3). The chocolate with 0.05% of almond did not amplify, confirming the qualitative PCR results (Fig. 1). The 10% almond extract was the only producing 6 positive replicates, unlike others, which suggests poor reproducibility (Table 3). The Wizard with RNase protocol enabled positive DNA amplifications until 0.05% with PCR efficiency of 106.4% and linear correlation of 0.958. However, chocolates containing 0.1% and 0.05% of almond only presented two or one positive replicates, with very high cycle threshold (Ct) values (Table 3).

DNA extracted with the Wizard Magnetic method presented the worst performance by real-time PCR (Table 3). Amplification was achieved only for chocolates containing 10% and 0.05% of almond, with the highest Ct values and the last concentration presenting one positive replicate. All the proportions in-between failed to amplify, agreeing with the qualitative PCR results (Fig. 1).

The protocols presenting the best real-time PCR results corresponded to CTAB-PVP and Nucleospin, being in good agreement with qualitative PCR results (Fig. 1). Both protocols allowed amplifying almond DNA from all the tested concentrations, with high PCR efficiency (90.4% and 98.5%) and linear correlation (R^2=0.959 and 0.975) (Table 3). Comparing their performances, the significantly lower DNA yields of CTAB-PVP method might be responsible for the inferior reproducibility. This can be noticed in the lowest tested level of 0.01%, from which only two replicates were amplified with the CTAB-PVP method. Ct values for this method ranged from 30.33 to 42.18, whereas level of amplification improved with Nucleospin method that enabled the significantly lowest Ct values (27.81 to 37.94). Hence, Nucleospin method was the chosen protocol to isolate almond DNA from model chocolates.

In view of the best performance of Nucleospin method, a wider range of spiking almond in chocolate was further tested by real-time PCR. Results of two independent PCR runs showed a LOD of 0.005%, with high PCR efficiency and linear correlation (Fig. 2a). LOD value was defined considering at least 95% of positive replicates per analytical run (Mazzara et al., 2008; Bustin et al., 2009), although positive amplifications were also verified at lower spiking levels (5 and 10 mg kg^{-1}). The limit of quantification (LOQ) was considered the lowest amplifiable concentration within the linear range, which in this case was equal to the LOD value.

Hazelnut

Analysis of the extracted DNA

Table 2 presents the estimated results for DNA concentration and purity of hazelnut spiked in chocolate extracted with the referred seven extraction protocols. The CTAB-PVP method produced the lowest DNA yields, though with acceptable purity (A_{260}/A_{280}=1.52), which is in accordance with previous results from almond chocolates. The Wizard protocols, with and without RNase, also produced extracts with significantly low DNA contents, but with the best purities. The Wizard Magnetic method enabled higher DNA yields from hazelnut in chocolates, when compared to the extracts from almond chocolates, although revealing lower purity (A_{260}/A_{280}=1.40). The extracts obtained with Wizard-PVP, Wizard-PVP with RNase and Nucleospin protocols revealed similar DNA yields, ranging from 82.4 to 98.1 ng μL^{-1} and purities within 1.31 until 1.36 (Table 2). The overall comparison of DNA yield and purity shows a high proximity between the two nuts within each extraction protocol.

Table 3. Real-time PCR amplification data from almond or hazelnut DNA of model chocolates obtained with different extraction methods

	Ct (mean ± SD)*							
	Amount of tree nut in chocolate					Real-time PCR performance parameters		
Extraction Method	10%	1%	0.1%	0.05%	0.01%	Slope	Efficiency (%)	R^2
Almond								
Wizard	32.33 ± 0.12c (6/6)**	36.68 ± 0.25b (5/6)	39.21 ± 1.25 (4/6)	na	41.42 ± 0.82 (2/6)	-3.047	112.9	0.956
Wizard with RNase	34.14 ± 0.11b (6/6)	38.27 ± 0.84a (4/6)	40.94 ± 0.59 (2/6)	40.97 (1/6)	na	-3.178	106.4	0.958
Wizard Magnetic	37.14 ± 0.37a (6/6)	na***	na	43.33 (1/6)	na	-2.689	135.5	0.991
CTAB-PVP	30.33 ± 0.19d (6/6)	33.17 ± 0.38c (6/6)	36.67 ± 0.67 (6/6)	37.92 ± 1.02 (6/6)	42.18 ± 0.97 (2/6)	-3.577	90.4	0.969
Nucleospin	27.81 ± 0.12e (6/6)	32.09 ± 0.25c (6/6)	34.89 ± 0.25 (6/6)	35.90 ± 1.03 (6/6)	37.94 ± 0.91 (6/6)	-3.359	98.5	0.975
Hazelnut								
Wizard-PVP	31.74 ± 0.49a (6/6)	34.57 ± 0.23a (4/6)	39.22 ± 0.71 (2/6)	39.51 (1/6)	na	-3.500	93.1	0.978
Wizard-PVP with RNase	30.77 ± 0.25b (6/6)	34.12 ± 0.17a (6/6)	37.31 ± 0.65 (2/6)	38.06 (1/6)	na	-3.217	104.6	0.996
Wizard	30.32 ± 0.20bc (6/6)	34.41 ± 0.31a (6/6)	37.34 ± 0.37 (4/6)	39.19 ± 0.33 (6/6)	40.36 ± 0.88 (2/6)	-3.524	92.2	0.984
Wizard with RNase	29.75 ± 0.16c (6/6)	33.07 ± 0.27b (6/6)	37.17 ± 0.70 (4/6)	37.43 ± 0.68 (3/6)	na	-3.562	91.2	0.985
Wizard Magnetic	30.44 ± 0.18bc (6/6)	33.78 ± 0.18ab (6/6)	36.87 ± 0.78 (4/6)	na	na	-3.224	104.2	0.987
CTAB-PVP	28.38 ± 0.03d (6/6)	31.43 ± 0.52c (6/6)	35.89 ± 0.08 (6/6)	37.36 ± 0.31 (5/6)	na	-3.892	80.7	0.984

*Values represent mean and standard deviation (SD). **In brackets are presented the numbers of positive replicates in n=6. ***na - no amplification. In each column, different letters indicate significant differences among DNA extraction methods ($p < 0.05$).

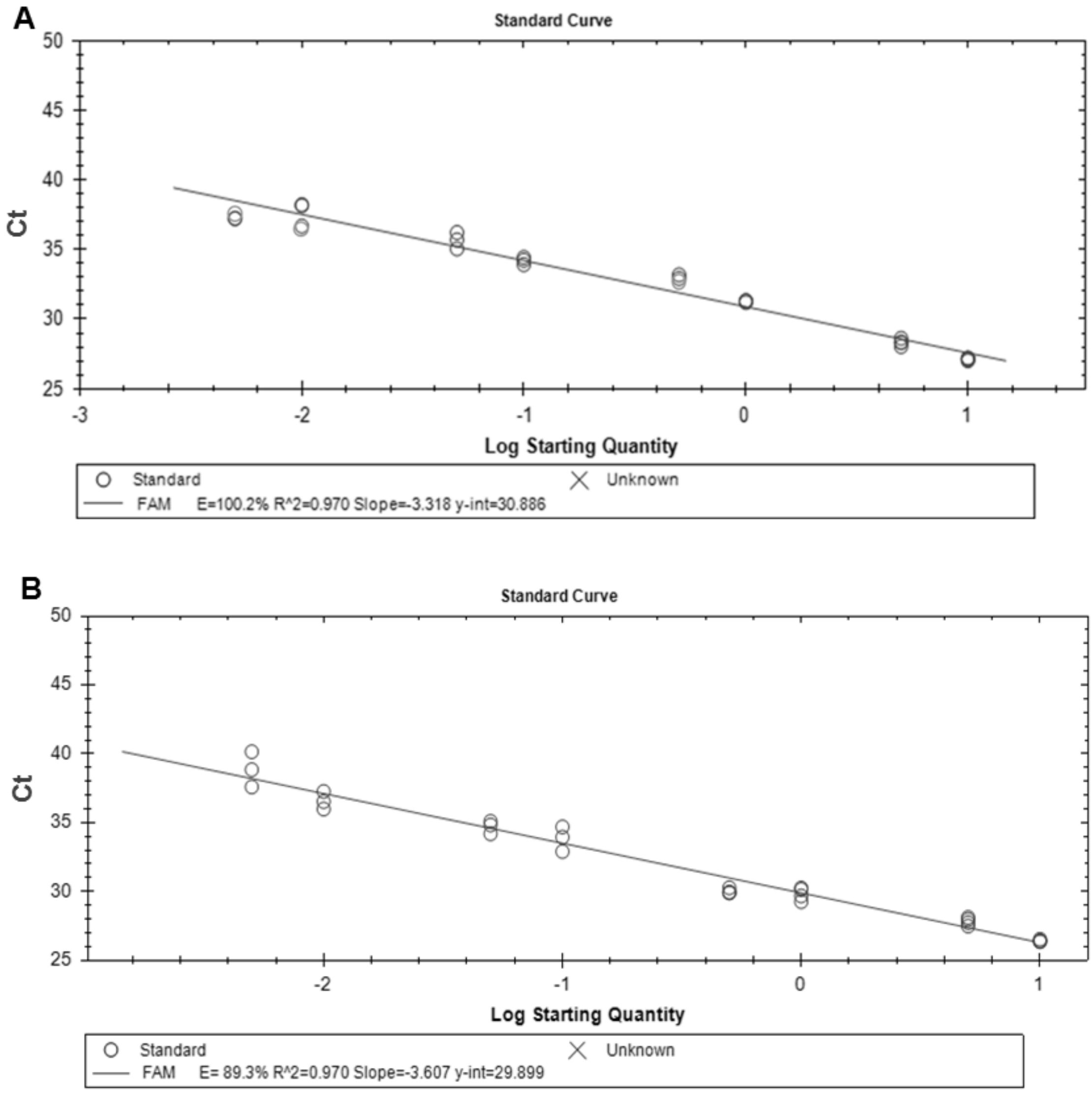


Fig. 2. Calibration curves obtained by real-time PCR amplification (n=4) with primers Prd6-1F/Prd6-1R and probe Prd6-1P (a) and with primers Nocc2F/Nocc1R-bis and probe Nocc2P (b) of model chocolates containing 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005% of almond or hazelnut, respectively, extracted with the Nucleospin method.

Qualitative PCR detection

Fig. 1 presents the PCR amplification results targeting a 100 bp fragment of the *hsp1* gene of hazelnut in chocolate extracted with different methods. Wizard-PVP protocol produced the expected fragments until the level of 0.05%, exhibiting a better sensitivity than the obtained for almond chocolates. The addition of RNase to the former protocol produced similar PCR fragments for the first three spiking levels, but decreased sensitivity to 0.1%. The Wizard method enabled the amplification of DNA fragments up to 0.05%, nevertheless with weak bands and without forming a clear gradient with decreasing hazelnut concentration. The addition of RNase to the Wizard protocol enhanced the quality of amplification, being all the tested levels positive for the target fragment. This result indicated a higher adequacy of this protocol for hazelnut DNA extraction than for almond in chocolate matrix.

The Wizard Magnetic extracts of hazelnut chocolate produced positive amplification in three concentration levels (10-0.1%). The same results were also achieved with CTAB-PVP protocol, which previously presented better sensitivity for almond-specific amplification. Nucleospin method enabled amplifying all standards with a clear gradient and a sensitivity of 0.01%, which is in good agreement with the results for almond in chocolate, exhibiting the best performance for qualitative PCR.

Real-time PCR detection

As for almond, real-time PCR results targeting hazelnut are summarised in Table 3. Wizard-PVP methods with and without RNase presented similar results, showing high PCR efficiencies (93.1% and 104.6%) and linear correlations (R^2=0.978 and 0.996). Sensitivities were also similar (0.05%), but inadequate due to the lack of reproducible replicate amplification, suggesting that the extracts did not reach the satisfactory purity for amplification by real-time PCR.

Extracts obtained with Wizard method allowed amplifying hazelnut from all tested spiking levels with high PCR efficiency (92.2%) and linear correlation (R^2=0.984), though with no reproducible amplification for concentrations ≤0.1%. Ct values were close or no significantly different from the two Wizard-PVP protocols, suggesting the presence of PCR inhibitors as verified in qualitative PCR (Fig.1). Wizard with RNase protocol maintained the performance of real-time PCR with similar or significantly lower Ct values comparing with the former methods. In spite of that improvement, the sensitivity only reached the level of 0.05% with lack of reproducible amplification, in opposition to the previous qualitative PCR results (Fig. 1).

The Wizard Magnetic method revealed significant improvements for hazelnut amplification comparing with almond, with high PCR efficiency (104.2%) and linear correlation (R^2=0.987), and Ct values close to the former methods. However, the sensitivity (0.1%) was the worst from all the tested protocols for hazelnut in chocolate.

CTAB-PVP method revealed a good performance by real-time PCR, achieving a sensitivity of 0.05% with PCR efficiency of 80.7% and linear correlation of 0.984. Comparing with the former methods, the Ct values of the two highest spiking levels were significantly reduced, suggesting improved purity for real-time PCR amplification that was also evidenced in the achieved reproducibility.

The Nucleospin method exhibited the best performance since it was the only one able to reach the highest sensitivity (0.01%), with high reproducibility and significantly lower Ct values (or similar to CTAB-PVP), comparing with all tested protocols. The referred improvements were achieved maintaining the adequate performance parameters of PCR efficiency (82.7%) and linear correlation (R^2=0.975).

Like for almond chocolates, the Nucleospin method seemed to be most suitable protocol to isolate hazelnut DNA from chocolate matrix. The application of real-time PCR to a wider range of spiked hazelnut in chocolate extracted with Nucleospin method allowed achieving a LOD of 0.005%, also with high PCR efficiency and linear correlation (Fig. 2b). The LOQ was equal to the LOD value since it was within the linear concentration range.

DISCUSSION

A reliable DNA extraction method is the basis for accurate allergen detection in complex and processed food matrices, such as chocolates. Regarding the extraction of amplifiable DNA from almond in chocolates, the Nucleospin method exhibited the best performance both by qualitative and real-time PCR, followed by the CTAB-PVP method. The later was considered the second best protocol with close behaviour to Nucleospin method, but with the disadvantage of low DNA yield that could compromise reproducibility and contribute to a higher variability among extractions. The addition of PVP to the CTAB lysis buffer intended to decrease contaminants such as polyphenols, being a possible reason for the successful application of this method for the extraction of DNA from chocolates. In the same way, other authors also introduced small alterations to the CTAB method, such as the use of mussel glycogen (Röder et al., 2011) and/or the use of higher amounts of starting material (Gryson, Messens, & Dewettinck, 2004), aiming at enhancing the effective extraction of DNA from different food matrices.

For the extraction of amplifiable DNA from hazelnut in model chocolates, Nucleospin method also revealed the best results in terms of qualitative and real-time PCR. Similarly to extraction of almond in chocolate, Nucleospin presented the highest reproducibility for hazelnut amplification by both PCR techniques. The second best protocol for hazelnut extraction was also the CTAB-PVP due to the higher reproducibility when compared to the Wizard method, which was reported in the literature as producing good results for DNA extraction from chocolates (Pafundo et al., 2010) and other food matrices (Fernandes, Oliveira, & Mafra, 2013; Mafra et al., 2008; Soares, Amaral, Mafra, & Oliveira, 2010a; Soares, Mafra, Amaral, & Oliveira, 2010b). However, none of those studies established the effectiveness regarding level of amplification by both qualitative and real-time PCR techniques. In the protocols based on Wizard and Wizard-PVP methods, the addition of RNase failed to improve the DNA extraction of almond or hazelnut from chocolate, being considered the methods with worst reproducibility. It is important to stress that the addition of RNase was crucial for the successful extraction of chocolate matrices with Nucleospin

(Costa et al., 2012b). Considering the applicability results of Wizard Magnetic method to both matrices, it can be inferred that it is not adequate for the isolation of amplifiable DNA from chocolates.

In this work, a relative LOD of 0.005% for both nuts spiked in chocolates was effectively attained using the Nucleospin method. In comparison with other described real-time PCR detection systems applied to chocolate matrices, the achieved level was apparently higher since Röder et al. (2011) and D'Andrea et al. (2009) reported 5 mg kg^{-1} for almond and 10 mg kg^{-1} for hazelnut, respectively. However, both authors did not follow the criteria of 95% of positive amplifications for the real-time PCR determination of the LOD (Mazzara et al., 2008; Bustin et al., 2009). In the present study, the reached sensitivities agreed with the reported values since amplifications for 5 and 10 mg kg^{-1} of both nuts in chocolates were also succeeded, but only in approximately 50% of the replicates (data not shown). As far as we know, other reports for the detection of nuts in spiked chocolates do not follow the recommended criteria by official entities, such as the European Network for GMO Laboratories, regarding the development of real-time PCR systems.

In summary, with this study it can be inferred that from the tested DNA extraction methods, the Nucleospin demonstrated to be the most suitable for quantitative real-time PCR amplification applied to both nuts in chocolate. To our knowledge this was the first report comparing different DNA extraction methods for the specific detection of different nut ingredients in chocolates. The presented results emphasize the importance of the DNA extraction protocol in the particular case of food allergens since sensitivity, a key parameter, depends on it. This work may also contribute to the development of highly reliable DNA-based methods as specific tools for the control of cross-contamination in complex food matrices along industrial processing, especially for chocolate manufacturers. Even so, more efforts are still required to improve the levels of DNA detection for quantitative purposes, which is of extreme importance for allergen management.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011 and by the University of Porto through "Projectos Pluridisciplinares" IJUP2011-176. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

Akkerdaas, J. H., Wensing, M., Knulst, A. C., Stephan, O., Hefle, S. L., Aalberse, R. C., et al. (2004). A novel approach for the detection of potentially hazardous pepsin stable hazelnut proteins as contaminants in chocolate-based food. *Journal of Agricultural and Food Chemistry*, *52*, 7726-7731.

Arlorio, M., Cereti, E., Coisson, J. D., Travaglia, F., & Martelli, A. (2007). Detection of hazelnut (*Corylus* spp.) in processed foods using real-time PCR. *Food Control, 18*, 140-148.

Bettazzi, F., Lucarelli, F., Palchetti, I., Berti, F., Marrazza, G., & Mascini, M. (2008). Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. *Analytica Chimica Acta*, *614*, 93-102.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry, 55*, 611-622.

Costa, J., Mafra, I., & Oliveira, M. B. P. P. (2012a). High resolution melting analysis as a novel approach for the detection of Pru du 5 almond allergen in foods. *Food Chemistry*, *133*, 1062-1069.

Costa, J., Mafra, I., Kuchta, T., & Oliveira, M. B. P. P. (2012b). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry, 60,* 8103-8110.

Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Novel approach based on single-tube real-time PCR to detect almond allergens in foods. *Food Research International*, *51*, 228-235.

D'Andrea, M., Coisson, J. D., Travaglia, F., Garino, C., & Arlorio, M. (2009). Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (*Corylus avellana* L.) in foods through calibration via plasmid reference standard. *Journal of Agricultural and Food Chemistry*, *57*, 11201-11208.

Fernandes, T. J. R., Oliveira, M. B. P. P., & Mafra, I. (2013). Tracing transgenic maize as affected by bread making process and raw material for the production of a traditional maize bread, broa. *Food Chemistry, 138,* 687-692,

Gryson, N., Messens, K., & Dewettinck, K. (2004). Evaluation and optimisation of five different extraction methods for soy DNA in chocolate and biscuits. Extraction of DNA as a first step in GMO analysis. *Journal of the Science of Food and Agriculture*, *84*, 1357-1363.

Holzhauser, T., & Vieths, S. (1999). Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *Journal of Agricultural and Food Chemistry*, 47, 4209-4218.

Holzhauser, T., Wangorsch, A., & Vieths, S. (2000). Polymerase chain reaction (PCR) for detection of potentially allergenic hazelnut residues in complex food matrixes. *European Food Research and Technology, 211*, 360-365.

Koppelman, S. J., Knulst, A. C., Koers, W. J., Penninks, A. H., Peppelman, H., Vlooswijk, R., et al., (1999). Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *Journal of Immunological Methods*, *229*, 107-120.

Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., & Waiblinger, H.-U. (2010). Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *European Food Research and Technology*, *230*, 367-374.

Krska, R., Becalski, A., Braekevelt, E., Koerner, T., Cao, X.-L., Dabeka, R., et al. (2011). Challenges and trends in the determination of selected chemical contaminants and allergens in food. *Analytical and Bioanalytical Chemistry*, *402*, 139-162.

Mafra, I., Silva, S. A., Moreira, E. J. M. O., Ferreira da Silva, C. S., & Oliveira, M. B. P. P. (2008). Comparative study of DNA extraction methods for soybean derived food products. *Food Control*, *19*, 1183-1190.

Mazzara, M., Savini, C., Delobel, C., Broll, H. Damant, A., Paoletti, C. et al. (2008). Definition of minimum performance requirements for analytical methods of GMO testing. European Network of GMO Laboratories (ENGL). Joint Research Center, European Commission (DOI: 10.2788/65827).

Mustorp, S. L., Drømtrop, S. M., & Holck, A. L. (2011). Multiplex, quantitative, ligation-dependent probe amplification for determination of allergens in foods. *Journal of Agricultural and Food Chemistry*, *59*, 5231-5239.

Pafundo, S., Gullì, M., & Marmiroli, N. (2010). Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. *Analytical and Bioanalytical Chemistry*, 396, 1831-1839.

Piknová, L., Pangallo, D., & Kuchta, T. (2008). A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. *European Food Research and Technology*, *226*, 1155-1158.

Poms, R. E., Klein, C. L., & Anklam, E. (2004). Methods for allergen analysis in food: a review. *Food Additives and Contaminants*, *21*, 1-31.

Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004, *Official Journal of European Union*, *L304*, 18-63.

Rejeb, S. B., Abbott, M., Davies, D., Cléroux, C., & Delahaut, P. (2005). Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Additives and Contaminants*, *22*, 709-715.

Röder, M., Vieths, S., & Holzhauser, T. (2011). Sensitive and specific detection of potentially allergenic almond (*Prunus dulcis*) in complex food matrices by TaqMan® real-time polymerase chain reaction in comparison to commercially available protein-based enzyme-linked immunosorbent assay. *Analytica Chimica Acta*, *685*, 74-83.

Sampson, H. A. (2003). Anaphylaxis and emergency treatment. *Pediatrics*, *111*, 1601-1608.

Schöringhumer, K., Redl, G., & Cichna-Markl, M. (2009). Development and validation of a duplex real-time PCR method to simultaneously detect potentially allergenic sesame and hazelnut in food. *Journal of Agricultural and Food Chemistry, 57*, 2126-2134.

Sicherer, S. H., & Sampson, H. A. (2006). Food allergy. *Journal of Allergy and Clinical Immunology*, *117*, S470-S475.

Soares, S., Amaral, J. S., Mafra, I., & Oliveira, M. B. P. P. (2010a). Quantitative detection of poultry meat adulteration with pork by a duplex PCR assay. *Meat Science, 85*, 531-536.

Soares, S., Mafra, I., Amaral, J. S., & Oliveira, M. B. P. P. (2010b). A PCR assay to detect trace amounts of soybean in meat sausages. *International Journal of Food Science & Technology, 45,* 2581-2588.

CHAPTER 2. HAZELNUT

STATE-OF-THE-ART

Hazelnut allergens: molecular characterisation, detection and clinical relevance

Critical Reviews in Food Science and Nutrition (accepted)

EXPERIMENTAL PART

Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut

Journal of Agricultural and Food Chemistry, **2012**, 60, 8103-8110

Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates

Food Chemistry, (submitted)

Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR

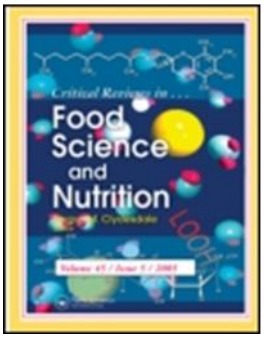
Analytical and Bioanalytical Chemistry (submitted)

STATE-OF-THE-ART

Hazelnut allergens: molecular characterisation, detection and clinical relevance

Critical Reviews in Food Science and Nutrition (accepted)

(accepted)



Critical Reviews in Food Science and Nutrition

Hazelnut allergens: molecular characterisation, detection and clinical relevance

Joana Costa[1], Isabel Mafra*[,1], Isabel Carrapatoso[2], Maria Beatriz P.P. Oliveira[1]

[1]REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal. [2]Serviço de Imunoalergologia, Centro Hospitalar e Universitário de Coimbra, Portugal.

*Corresponding author: Tel: +351 220428640. Fax: +351 226093390. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

In the last years, special attention has been devoted to food-induced allergies, from which hazelnut allergy is highlighted. Hazelnut is one of the most commonly consumed tree nuts, being largely used by the food industry in a wide variety of processed foods. It has been regarded as a food with potential health benefits, but also as a source of allergens capable of inducing mild to severe allergic reactions in sensitised individuals. Considering the great number of reports addressing hazelnut allergens, with an estimated increasing trend, this review intends to assemble all the relevant information available so far on the main issues: prevalence of tree nut allergy, clinical threshold levels, molecular characterisation of hazelnut allergens (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 10, Cor a 11, Cor a 12, Cor a 14 and Cor a TLP) and their clinical relevance, and methodologies for hazelnut allergen detection in foods. A comprehensive overview on the current data about the molecular characterisation of hazelnut allergens is presented, relating biochemical classification and biological function with clinical importance. Recent advances on hazelnut allergen detection methodologies are summarised and compared, including all the novel protein- and DNA-based approaches.

Keywords: Food allergens, hazelnut allergy, *Corylus avellana* L., prevalence, threshold levels, detection

INTRODUCTION

Hazelnut is classified as a tree nut that belongs to the botanical family of Betulaceae and to the genus *Corylus*. According to the United States Department of Agriculture-Germplasm Resources Information Network (USDA-GRIN), the genus *Corylus* encompasses more than 14 different species of hazels distributed all over the world (USDA, 2013). The nuts from all the hazel trees are considered edible, however the most cultivated and consumed correspond to the seeds of the hazel species *Corylus avellana* L., usually known as "common hazelnut". This hazel species is native from Europe and Western Asia (Caucasus region), but is also cultivated in North America. In general, alternative names such as cobnut or filbert are frequently used to designate common hazelnuts, although this classification is mostly attributed to the nuts of species *Corylus avellana* or *Corylus maxima*, respectively. Apart from those, the nuts from other hazel species can also be consumed, though they do not represent any relevant interest in terms of trade. There is a large number of hazel (*Corylus avellana* L.) cultivars and selections (USDA, 2013), being Morell, Negret, Grossal, Buttler, Ennis, Pauetet, Fertile de Coutard, Segorbe, Sta María del Gésu, Tonda di Giffoni, Culplà, Camponica, Cosford, Gunslebert, Lansing, Palaz, Sivri, Tombul and Tonda Romana, some examples of the varieties originated and/or cultivated in the south of Europe and Caucasus region.

The edible part of the hazelnut is the kernel, which can be consumed either raw or roasted (snacks), or included as an ingredient in a wide range of processed foods such as cakes, creams, chocolates and confectionary products (Alasalvar and Shahidi, 2008). Subsequently, hazelnuts along with other tree nuts play an important role in economy since they are an integral part of human food supply (Costa et al., 2012a). Among the worldwide production of tree nuts in 2011, hazelnut represented the seventh most relevant culture and, in terms of global trade, these seeds occupied the fourth position just behind pistachio, almond and cashew nut, respectively. In 2011, Western Asia and Europe retained almost 89% of the world's total production of hazelnut. The three major producers of this nut are Turkey, Italy and Azerbaijan, ranking the first, second and third places, respectively, since these countries alone are accountable for approximately 80% of hazelnut production in each year (FAOSTAT, 2013).

From some years, hazelnuts as well as other tree nuts have been regarded as foods with potential health benefits, namely as "heart-protective", which have led to an increase in their consumption, especially in the developed countries (FDA, 2003). Apart from this recognition, tree nuts have also been pointed as likely to induce hypersensitivity in sensitised/allergic individuals. Therefore in 1985, the Codex Alimentarius Commission recommended the obligation to label foods susceptive of containing potentially allergenic ingredients and since 1993, tree nuts are defined as one of the eight groups responsible for almost 90% of human food allergies (CODEX STAN 1). Accordingly, the European Union (EU) has established some directives determining the clear obligation of food producers to declare all the ingredients present in pre-packaged foods commercialised inside the EU (Directive 2000/13/EC). Presently, tree nuts are included in a list of fourteen groups of certain substances or products causing allergies or intolerances that are required to be emphasised from the rest of the list of ingredients of processed foods, regardless of their quantity (Directive 2007/68/EC, Regulation (EU) 1169/2011).

From all tree nuts, hazelnut is probably the most well studied nut, regarding the impact of its presence in the life of sensitised individuals. The purpose of this review is to provide an actual and critical overview on the prevalence of hazelnut allergy, the molecular characterisation of its allergens and the available methodologies for its detection. Herein, important subjects such as the clinical relevance of hazelnut allergy and the definition of threshold levels will also be addressed.

PREVALENCE OF TREE NUT ALLERGY: THE CASE OF HAZELNUT

Food-induced allergy represents an emerging problem of public health affecting adults and children, and whose prevalence is estimated to be rising. By definition, food allergy is a reproducibly adverse health effect arising from a specific immunological response that occurs in sensitised individuals upon exposure to a given food (Boyce et al., 2010). General data seem to indicate that the number of sensitised/allergic patients affected by food allergy is higher than 1-2%, but less than 10% of the world's population (Chafen et al., 2010). Although the available data is considered rather imprecise, some studies suggest that as many as 3-4% of adult population and 5-6% of young children/adolescents can suffer from some type of allergy related to food (Sicherer and Sampson, 2009; Sicherer and Sampson, 2010). In Europe alone, the number of food allergic patients is estimated to reach the 17 millions of individuals, which represents approximately 2.3% of the European population. From those, 3.5 million of the European allergic patients are younger than 25 years old, with the sharpest rise in food allergies being among children and young people (EAACI, 2012).

The true prevalence of food allergies has been very difficult to determine due to several inconsistences regarding key issues such as those related to study design. The majority of the information about the prevalence of food allergies is based on self-reported reactions to foods (questionnaires/surveys), rather than using objective assessments as open and double-blind food challenge tests, or determined sensitisation to foods by serum immunoglobulin E (IgE) and skin prick tests (SPT) (Zuidmeer et al., 2008). Consequently, prevalence data must be interpreted with caution and considered as mere indicators of the true incidence of food allergy.

In Europe, tree nuts are regarded as a common cause of food allergy (Ortolani et al., 2000), with hazelnut representing a significant part of those induced-allergies, while in USA, allergies to peanut and/or tree nuts such as almond, walnut or cashew, appear to be more relevant (Sicherer et al., 2003). In an eleven-year follow up self-reported study, conducted between 1997 and 2008 in the USA and encompassing three different surveys during this period (1997, 2002 and 2008), the prevalence of peanut and/or tree nut allergy was higher than 1.1%, which corresponded to more than three millions of individuals of the general US population. The same study also enabled to estimate that the increasing number of allergic patients to peanut and/or tree nut was more significant in individuals under 18 years-old and with walnut, cashew, pecan nut and almond, presenting the highest allergy incidence among the US population (Sicherer et al., 2010). Regarding the same age-target population, a randomised cross-sectional survey was administrated electronically to a representative sample of US households in 2009, which enable estimating an overall incidence of 8% of food allergic children (<18 years-old). According to this study the prevalence of nut allergy was approximately 1.0% with more than 52% of the allergic children suffering from severe immunological reactions (Gupta et al., 2011). Another study performed in Canada, using a nationwide telephone survey of randomly selected households in 2011, reported an overall incidence of approximately 6.7% of individuals with allergy to at least one food. The prevalence data for this region was higher for children (7.1%) than among adult population (6.6%). The estimated incidence of tree nut allergy was approximately 1.22% for the entire population and 1.74% for children, confirming the prediction of the overall indicators (Soller et al., 2012).

Usually, the prevalence studies involve only data from one country or region, thus in order to provide a broader overview about this topic, the European Commission funded in 2005 a large Europe-wide research project (EuroPrevall) specifically designated to evaluate the prevalence, basis and cost of food allergies. This project included the participation of 56 partners from 21 countries, being from 19 European countries, Ghana and India, as well as additional co-partners from USA, Australia and New Zealand (Mills et al., 2007a). Study designs involved birth cohorts, community surveys and outpatient clinical studies, with complementary information provided by SPT and double-blind placebo-controlled food challenges (DBPCFC). Part of the project tasks also covered the evaluation of the prevalence data available in the literature concerning food-induced allergies. In this context and on the basis of the comparison of studies published during the past 15 years, Zuidmeer et al. (2008) reported a range of prevalence regarding nut allergies of 0.03-0.2% in children up to 6 years-old, 0.2-2.3% in children/adolescents between 6-18 years and 0.4-1.4% in adults. In accordance with challenge tests and sensitisation assessed by SPT, the highest prevalence of nut allergies was estimated for hazelnut (4%). Adolescents and adults seem to be more affected by nut allergies, probably due to their late introduction into the diet. As nuts are often eaten separately, they are more easily identified as the possible cause for the observable symptoms, rather than other fruits or vegetables that are usually consumed in mixed dishes, making it difficult to distinguish the allergenic ingredient (Zuidmeer et al., 2008). Included in the EuroPrevall project and involving several centres from a total of 13 countries (USA, Australia and eleven countries from Europe), sera from test subjects were scanned using 5 allergen mixes from a total of 24 foods previously defined as priorities. Allergy induced by hazelnut presented the highest overall incidence, accounting with approximately 7.2% of the test population (Burney et al., 2010). Hazelnut allergy is often related to birch pollinosis, therefore some patients are commonly allergic to the nut itself, others are allergic to the pollen of hazel trees, but frequently patients are allergic to both (Roux et al., 2003). These facts seem to be well stated in the study reported by Burney et al. (2010), as after excluding the birch positive patients, the percentage of positive reactants to hazelnut kernel was reduced to approximately 3.1%. USA presented the highest incidence (14.9%) of allergic patients to hazelnut, being closely followed by Germany (14.7%), Norway (12.8%), Switzerland (12.6%) and Sweden (11.8%). From the panel of 24 foods, hazelnut was also considered the food with the highest prevalence of allergy at least in 46% of the participating countries (Burney et al., 2010).

From all the exposed information, it is clear that more studies aiming at establishing the prevalence of tree nut allergies are still needed, specially focusing on each of the most significant nuts.

CLINICAL THRESHOLD LEVELS FOR HAZELNUT

Presently, the only effective means of preventing any adverse immunological reactions in the allergic patients is based on the total avoidance of the offending foods. As a consequence of this protective measure, those individuals face several restrictions when carefully choosing processed foods that are commercially available. In order to avoid probable legal actions against food-processing companies, products' labels are often excessively precautionary due to the potential risk of cross-contaminations in the production line or during storage. Thereby, the definition of clinical threshold values for allergenic foods would be of utmost interest since it could contribute to a better risk management by the regulatory authorities, providing more adequate guidelines to food industry. Not less relevant, information about personal thresholds would enable patients, caretakers and physicians to establish adequate individual strategies aiming at preventing potential adverse immunological responses (Eller et al., 2012). The term threshold is attributed to the dose of the allergenic food that lies between the highest amount of the offending food not eliciting any allergic response and the lowest observed adverse effect level (LOAEL). In general, the threshold dose is functionally defined as the LOAEL or the no observed adverse effect level (NOAEL), being determined either on an individual or on a population basis (Taylor et al., 2009). The assessment of individual NOAEL and LOAEL parameters can be performed by clinical challenge trials such as open food challenges (OFC) and DBPCFC. In practice these challenges cannot be carried out in all food-allergic patients. However, statistical models based on individual LOAEL have been conducted for the elaboration of a dose-response curve for a given allergen (Crevel et al., 2007).

Using clinical challenge tests (OFC and DBPCFC) on a large test population (487 food-allergic patients), Eller et al. (2012) was able to establish threshold values for hazelnut, egg, peanut and milk. In the case of hazelnut, the frequency of first-dose responders with objective symptoms corresponded to 8% of the test group when an initial dose of 1 mg was administrated to those patients. Therefore, this dose (1 mg) was considered the lowest amount of allergenic food inducing observed adverse immunological responses and consequently defined as the threshold value for hazelnut. In the same study, those authors were able to predict that 8.7 mg and 15.9 mg of hazelnut protein were sufficient to induce allergic reactions in 5% and 10% of the hazelnut-allergic population, respectively (Eller et al., 2012). Another study estimating the threshold distribution of seven allergenic foods (egg, milk, peanut, hazelnut, walnut, cashew nut and soy) was conducted using DBPCFC in children and adolescents (0-18 years-old) as test-population (Blom et al., 2013). In this study, patients with hazelnut allergy were the most sensitised group since 5% and 10% of the tested population is likely to respond with objective symptoms to 0.3 mg (ED_{05}) and 1.4 mg (ED_{10}) of hazelnut protein, respectively. Moreover, the same target population evidenced any type of symptoms for the estimated threshold level of 0.05 mg of hazelnut protein (Blom et al., 2013).

From the evaluation of these two studies, it is very clear the influence of different parameters such as the size, age and sex of the target allergic population as well as the type of food matrices and the type of food challenges used (OFC versus DBPCFC). The distribution of the threshold level for hazelnut reported by those studies is rather different, which suggests that further investigation regarding this topic is still needed. However, these data represent a step forward in the elaboration of precautionary labelling action levels and for the incorporation in the risk assessment for adverse immunological reactions in the allergic population, upon eventual consumption of foods contaminated with allergens (Blom et al., 2013).

MOLECULAR CHARACTERISATION OF HAZELNUT ALLERGENS

The majority of the molecules defined as food allergens are biochemically classified as proteins or glycoproteins that are naturally present in foods (Boyce et al., 2010). In the specific case of the hazelnut, several proteins have been recognised as allergens. Until now, ten groups of allergenic proteins (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 10, Cor a 11, Cor a 12, Cor a 13, Cor a 14 and Cor a TLP) have been identified and characterised in hazelnut. From those, Cor a TLP has not yet been comprised in the WHO-IUIS list of allergens (ALLERGEN, 2013), but has already been integrated in the Allergome database (ALLERGOME, 2013). All the other hazelnut allergens are included in the referred list with the classification of food allergens, with an exception for Cor a 10 since it is only present in the pollen of hazel trees (ALLERGEN, 2013).

Cor a 1 (PR-Proteins)

The pathogenesis-related proteins comprise a collection of several unrelated families that are expressed in response to external factors such as environmental stress, pathogen infection or antibiotic stimuli. These proteins are characterised by their small size, stability at low pH and resistance to proteolysis, making them good candidates for inducing adverse immunological responses in sensitised individuals (Hauser et al., 2008). The PR-10 proteins are included in the PR protein superfamily, being commonly known as the Bet v 1-related proteins or Fagales group I because they are very common among the trees from the order Fagales. Cor a 1 allergens are classified as PR-10 proteins, which are greatly abundant in the reproductive tissues such as pollen, fruits and seeds. As consequence, Cor a 1 proteins are classified both as food allergens and as aeroallergens.

Cor a 1 appear to comprise a complex set of proteins encoded by different nucleotide sequences ranging from 486 to 860 base pairs (bp) (NCBI, 2013), presenting distinct allergenicity among proteins (Lüttkopf et al., 2001). Cor a 1 exhibit four isoallergens designated by Cor a 1.01, Cor a 1.02, Cor a 1.03 in hazel pollen, and Cor a 1.04 in hazelnut seed, with molecular weights of approximately 17 kDa (Table 1) (Roux et al., 2003) These four sequences are denominated isoallergens due to their similar molecular size, identical biological function and 67% or more, amino acid identity (Chapman et al., 2007). Isoallergen Cor a 1.04 comprise four variants or isoforms (Cor a 1.0401-Cor a 1.0404) with 161 amino acids (aa), a calculated molecular mass of 17.4 kDa and an isoelectric point (pI) of 6.1 (Lüttkopf et al., 2001).

Table 1 Identification of almond allergens according to their biological function, clinical relevance and respective accession numbers in the NCBI and UniProt databases

Allergen	Molecular Weight (kDa)	Isoallergens	Isoforms or variants	Nucleotide (NCBI)	Protein (NCBI)	Protein (UniProt)	Protein Families	Biochemical classification	Biological Function	Clinical relevance
Cor a 1	17 (160 aa)	Cor a 1.01	Cor a 1.0101	X70999.1	CAA50327.1	Q08407	Pathogenesis-Related Proteins	PR-10	Involved in mechanisms of defence and biotic stimulus responses	Mild symptoms mostly related to OAS. (Major allergen)
			Cor a 1.0102	X71000.1	CAA50328.1	Q08407				
			Cor a 1.0103	X70997.1	CAA50325.1	Q08407				
			Cor a 1.0104	X70998.1	CAA50326.1	Q08407				
		Cor a 1.02	Cor a 1.0201	Z72439.1	CAA96548.1	Q39453				
		Cor a 1.03	Cor a 1.0301	Z72440.1	CAA96549.1	Q39454				
	(161 aa)	Cor a 1.04	Cor a 1.0401	AF136945.1	AAD48405.1	Q9SWR4				
			Cor a 1.0402	AF323973.1	AAG40329.1	Q9FPK4				
			Cor a 1.0403	AF323974.1	AAG40330.1	Q9FPK3				
			Cor a 1.0404	AF323975.1	AAG40331.1	Q9FPK2				
Cor a 2	14 (131 aa)	Cor a 2.01	Cor a 2.0101 Cor a 2.0102	AF327622.1 AF327623.1	AAK01235.1 AAK01236.1	Q9AXH5 Q9AXH4	Profilin	Profilin	Binds to actin and affects the structure of the cytoskeleton.	Mild symptoms mostly related to OAS. (Minor allergen)
Cor a 8	9 (115 aa)	Cor a 8.01	Cor a 8.0101	AF329829.1	AAK28533.1	Q9ATH2	Prolamin	PR-14	Transference of phospholipids and galactolipids across membranes. Antimicrobial activity.	Severe and systemic reactions. (Major allergens)
Cor a 9	40 (515 aa)	Cor a 9.01	Cor a 9.0101	AF449424.1	AAL73404.1	Q8W1C2	Cupin	11S seed storage globulin (legumin-like)	Storage of nutrients for plant growth	Severe and systemic reactions. (Major allergens)
Cor a 10	70 (668 aa)	Cor a 10.01	Cor a 10.0101	AJ295617.1	CAC14168.1	Q9FSY7	heat shock protein 70	Luminal binding protein	Interacting selectively and non-covalently with ATP (binds ATP and nucleotides)	No data available
Cor a 11	48 (448 aa)	Cor 11.01	Cor a 11.0101	AF441864.1	AAL86739.1	Q8S4P9	Cupin	7S seed storage globulin (vicilin-like)	Storage of nutrients for plant growth	Severe and systemic reactions (Major allergens but classification should be revised)
Cor a 12	17 (159 aa)	Cor 12.01	Cor a 12.0101	AY224679.2	AAO67349.2	Q84T21	Oleosin	17 kDa oleosin	Cellular component. Intervene in lipid metabolism and storage, regulation of intracellular trafficking and signal transduction.	No data available. (Suggested major allergen classification)
Cor a 13	14-16 (140 aa)	Cor 13.01	Cor a 13.0101	AY224599.1	AAO65960.1	Q84T91	Oleosin	14-16 kDa oleosin	The same as Cor a 12	No data available. (Suggested major allergens classification)
Cor a 14	15-16 (147 aa)	Cor 14.01	Cor a 14.0101	FJ358504.1	ACO56333.1	D0PWG2	Prolamin	2S albumin	Storage of nutrients for plant growth	Moderated to severe symptoms. (Minor allergens)
Cor a TLP	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Pathogenesis-Related Proteins	Thaumatin-like protein (PR-5)	Anti-fungal activity	No data available (Suggested minor allergens classification)

They are polymorphic variants of the same allergen (Chapman et al., 2007) and among them exhibit 97-99% of amino acid identity with a maximum of six substitutions in five of the highly conserved regions. However, they only share 63% or more sequence identity with the other six hazel pollen isoforms (Table 1) (Lüttkopf et al., 2001). The amino acid sequence identity is higher between Cor a 1.04 and Bet v 1 (85%), than with the other pollen isoallergens (Cor a 1.01, Cor a 1.02 and Cor a 1.03) from the same tree. This fact seems to suggest that in some populations, the majority of the patients can be primarily sensitised to birch pollen (Bet v 1) (Roux et al., 2003). Cor a 1.01, which is classified as hazel pollen isoallergen, also comprises four isoforms (Cor a 1.0101 to Cor a 1.0104) with sequence identity higher than 95% among variants.

In general, these allergens are considered to be heat-labile proteins, suggesting that they suffer unfolding when submitted to heat treatments such as food processing (Hansen et al., 2003; Müller et al., 2000; Pastorello et al., 2002; Schocker et al., 2000). The loss of the protein tri-dimensional structure enables the destruction of the conformational epitopes (IgE-reactivity) and, consequently, the ability to trigger adverse reactions in sensitised individuals (Mills et al., 2007b). Dry heat processing such as roasting enables reducing significantly the allergenicity of hazelnuts in patients with positive diagnosis for birch-pollen allergy related to hazelnuts, although some few sensitised individuals can still experience positive reactivity towards roasted hazelnuts (Hansen et al., 2003). Structure models for Cor a 1 proteins revealed that most part of the amino acid sequence is well organised, with loops located almost exclusively at the apical structures of beta-turns and thus being very influenced by the beta arrangement itself. López et al. (2012) reported that the application of autoclave processing (121ºC or 138ºC, 15 or 30 min) to hazelnut samples allowed decreasing the allergenicity of Cor a 1, since the location of the epitopes is mainly dependent on the conformational structure of the protein, which is affected by heat treatment. This finding is in good agreement with data reported by Hansen et al. (2003), stating that Cor a 1 allergenicity is highly influenced by the tri-dimensional conformation of the proteins. As an alternative to thermal processing, new technologies such as high pressure processing have been exploited to evaluate their effect on the structural conformation of allergens and hence to decrease their allergenic activity (Mills et al., 2003). The application of high pressure processing with increasing pressures, ranging from 300 to 600 Mba, was performed aiming at testing its effect on the allergenicity of Cor a 1 proteins, though resultant allergenic profile remained the same as from raw hazelnuts (López et al., 2012).

Cor a 2 (Profilins)

Profilins are a family of cytosolic actin binding proteins with small molecular size (12-15 kDa) and constituted by polypeptides ranging from 124 to 153 aa (Vieths et al., 2002). They are highly conserved molecules, sharing more than 75% of amino acid sequence identity with profilins from members of distantly related organisms (Hauser et al., 2010). The sequence conservation reflected among all eukaryotic cells is evidenced by similar tertiary structures and the identical biological functions of profilins (Hauser et al., 2008). These proteins are important mediators of membrane-cytoskeleton communication, being able to specifically bind to ligands such as actin, phosphatidylinositol-4,5-bisphosphate (PIP2) and poly-L-proline (Vieths et al., 2002). Such characteristics enable profilins to actively intervene in processes associated with cell motility (regulation of the actin microfilament polymerisation) and interact with the PIP2 pathway of signal transduction (Valenta et al., 1992, Vieths et al., 2002). As components of many essential cellular processes, profilins are ubiquitously spread through nature. Therefore they can be considered as pan-allergens that are responsible for several of the observed cases of cross-reactivity between inhalant and food allergens (Valenta et al., 1992).

One group of allergens identified in hazelnut comprises a family of profilins named Cor a 2, which were characterised as a relevant IgE-binding protein for a minority of pollen-nut allergic individuals (Hirschwehr et al., 1992). Cor a 2 proteins are classified as pollen and food allergens since they are both present in the pollen of hazel trees and in their respective seeds/nuts (hazelnuts) (Hauser et al., 2010). Two variants of Cor a 2 allergens, encoded by nucleotide sequences of 396 bp, have been described (NCBI, 2013), namely Cor a 2.0101 and Cor a 2.0102 (Table 1). Allergen isoforms present sequences with 131 aa, similar molecular size (14.0 kDa and 14.1 kDa) and acidic proprieties (pI of 4.9 and 4.7) for Cor 2 a 2.0101 and Cor a 2.0102, respectively (Vieths et al., 2002). Sequence identity between the two variants (Cor a 2.0101 and Cor a 2.0102) is higher than 98%, and approximately 77% towards Bet v 2, which states that Cor a 2 is one of the Bet v 2-related allergenic food profilins. Similarly to isoallergen Cor a 1.04, the allergenicity of Cor a 2 seems to decrease with food processing (roasting), in patients sensitised to birch-pollen hazelnut allergens, suggesting that profilins Cor a 2 are also heat-labile proteins (Hansen et al., 2003).

Cor a 8 (nsLTP)

Cor a 8 is another group of allergenic proteins present in hazelnut, which belongs to the family of the non-specific lipid transfer proteins (nsLTP) that is included in the prolamin superfamily. Along with the family of the nsLTP, this superfamily comprises several groups of proteins that contain many plant food allergens such as the 2S albumins and inhibitors of alpha-amylase and trypsin from cereals (Breiteneder, 2006). The family of nsLTP is characterised by monomeric proteins of low molecular size, revealing primary sequences with a high content of cysteine residues, thus, contributing to secondary structures composed by alpha-helices that involve a lipid binding cavity in the core. Members from this family share common structural features that include eight cysteine residues bonded in four disulphide bridges, basic isoelectric points and high similarity in the amino acid sequences (Kader, 1996). One of the biological functions of nsLTP is related to their ability to transport different types of lipids (fatty acids, phospholipids, glycolipids and sterols) through membranes, comprising two subfamilies of 9 kDa proteins (nsLTP 1) or 7 kDa proteins (nsLTP 2), respectively (Hauser et al, 2010; UniProt, 2013). The nsLTP are also interveners in other functions such as those associated with plant defence (antifungal and antibacterial activities) (Ebner et al., 2001) or potential involvement in plant growth and development (embryogenesis, germination) (Kader, 1996; Salcedo et al., 2007), being widely distributed throughout the kingdom of plants (Hauser et al, 2010). According to these facts, the nsLTP were included in the class of pathogenesis-related proteins, representing the PR-14 family.

In hazelnut, Cor a 8 is classified as a food allergen due to its exclusive presence in the nutritive tissues (seed). With a polypeptide chain of 115 aa and a molecular weight of 9 kDa, Cor a 8 is encoded by a nucleotide sequence of 348 bp (NCBI, 2013). Considering that the immature protein contain a signal peptide of 23 aa, the mature Cor a 8 allergen presents a total of 92 aa (Schocker et al., 2004) with alpha-helical structure (Rigby et al., 2008). According to the NCBI database, upon protein alignment of relevant allergenic nsLTP from different species, Cor a 8 exhibited 60% of sequence identity with Pru du 3 (almond, ACN11576), 59% with Mal d 3 (apple, AAR22488) and Pru ar 3 (apricot, ADR66948), 58% with Pru av 3 (cherry, AAF26449), 56% with Pru p 3 (peach, ACE80969) and 54% with Pyr c 3 (pear, AAF26451) (NCBI, 2013), which indicates their structural relationship (Salcedo et al., 2007). Therefore, it is expected to occur IgE cross-reactivity between Cor a 8 and the allergens from fruits of the Rosaceae family (Schocker et al., 2004). Several factors are known to affect the allergenic potential of the nsLTP, namely the location of the proteins and the stability of those to thermal or proteolytic processing. In fruits from the Rosaceae family, the nsLTP are predominantly accumulated in the outer epidermal layers, being responsible for the stronger allergenicity of the peels in comparison with the inner pulps (Fernández-Rivas and Cuevas, 1999). Studies evidenced that some LTP-sensitised individuals can tolerate fruits (apple, peach) after peeling (Fernández-Rivas and Cuevas, 1999), however they are still at risk of developing adverse reaction upon the ingestion of nuts. The nsLTP are regarded as true food allergens considering that these molecules are capable of eliciting severe allergic responses after resisting to food processing (thermal treatments and abrupt pH changes) and to the inhospitable environment of the gastrointestinal tract (proteolysis) (Zuidmeer and van Ree, 2007). Like for other nsLTP, Cor a 8 was also found to be resistant to the activity of gastric and intestinal enzymes, which justify its capacity to induce severe allergic reactions in some sensitised individuals (Schulten et al., 2011a). In contrast with the 2S albumin family, the nsLTP are slightly less stable when submitted to temperatures over 90ºC, probably due to the existence of the lipid-binding tunnel (Mills et al., 2007b; Sancho et al., 2005). As for Cor a 1, the allergenicity of Cor a 8 was significantly affected when submitting hazelnuts to high temperatures and wet processing, such as autoclave (121ºC and 138ºC, for 15 and 30 min), since autoclaving induces the disorganisation of almost all possible epitopes in this protein. The application of high pressure processing (300 to 600 Mba) to hazelnut samples did not affect the IgE-binding capacity of Cor a 8 in the test population (López et al., 2012). Like the profilins, the nsLTP are also considered pan-allergens, suggesting that these proteins are well spread throughout the nature. The highly conserved regions and tri-dimensional structures seem to ensure the functionality of nsLTP, even those originating from unrelated sources, enabling to satisfy the requisites for IgE recognition (Hauser et al., 2010).

Cor a 9 (11S Globulin - Legumin)

Cor a 9 proteins constitute another group of hazelnut allergens that belong to the cupin superfamily. It encompasses a large and multifunctional variety of proteins sharing a common origin, as their evolution can be traced from bacteria to eukaryotes, including animals and superior plants (Dunwell et al., 2004; Hauser et al., 2008). Cupin superfamily comprises two functional classes of proteins, namely the monocupins and the bicupins, containing one or two conserved cupin domains, respectively. The dicupin class includes the 7S and 11S globular seed storage proteins, which represent major components of the human diet. In tree nuts as well as in several legumes, the seed storage globulins represent almost 50% of the total seed proteins that contain the resources needed for plant germination. Globulins are further divided in two groups, the 7S vicilin-type globulins and the 11S legumin-type globulins, according to their sedimentation coefficient (Breiteneder, 2006).

Cor a 9, also known as corylin, is classified as an 11S legumin-type globulin (Beyer et al., 2002; Guo et al., 2009). It is expressed by a gene with 1767 bp encoding a protein of 515 aa with a theoretical molecular mass of 59 kDa. Each isoform of 11S globulin is apparently coded by a single gene producing a precursor that is post-transnationally split in the asparaginyl endopeptidase site. The functional 11S legumins are non-glycosylated proteins, forming hexameric structures composed by six subunits interacting non-covalently and arranged in an open ring conformation with 360 kDa (Breiteneder, 2006). Each subunit is constituted by an acidic polypeptide (30-40 kDa) linked to a basic polypeptide (~20 kDa) by a disulphide bond. 11S Globulins from several tree nuts display from 8 to 15 linear epitope-bearing peptide regions that are scattered along the length of the acidic and the basic subunits (Robotham et al., 2009). Functional Cor a 9 is also composed of acidic and basic polypeptide chains linked by a disulphide bond (Beyer et al., 2002). The cleavage site in the peptide bond seems to be well conserved among a wide variety of plant species. Cor a 9 and several other legumins possess the NGXEET motif: NGFEET in Cor a 9 from hazelnut, NGLEET in Pru du 6 from almond and Jug r 4 from walnut, and NGIEET in Ana o 2 from cashew and Ara h 3 from peanut (Albillos et al., 2008). The identified allergen with molecular weight of 40 kDa is the acidic subunit of Cor a 9 after cleavage and reduction of the protein (Table 1) (Beyer et al., 2002). A BLAST search evidenced sequence identities ranging from 46% to 70% between Cor a 9 and other plant allergens such as Gly m 6 from soybean, Ara h 3 from peanut, Ana o 2 from cashew, Ber e 2 from brazil nut, Pru du 6 from almond and Pis v 2 from pistachio. Belonging to the legumins, Corylin (Cor a 9) also presents a hexameric form organised in a quaternary structure, which is in good agreement with the functional structure attributed to other 11S globulins, namely Pru du 6 from almond (Albillos et al., 2008). The IgE binging epitope(s) have not yet been identified however, Cor a 9 presents 67% of sequence identity with the corresponding region of the linear IgE binding epitope of peanut allergen Ara h 3 (Beyer et al., 2002).

Legumins are thermostable proteins, only suffering partial unfolding of their conformational structures at temperatures above 94ºC (Mills et al., 2007b). However, even after being submitted to high temperatures, the secondary structures of these proteins remain unchanged or with minor modifications, suggesting that the characteristic beta-barrel motif is highly stable. Preliminary studies on raw hazelnuts submitted to dry heat treatment at 170ºC, indicated that the protein profile and the amount of Cor a 9 was not affected until after 20 min of roasting (Dooper et al., 2008). More recently, López et al (2012) verified that Cor a 9 is a highly well-structured protein, enriched by a beta-sheet core and with long unstructured loops. Those loop regions are estimated not to be modelled due to their lack of stable structure, but they are predicted to exhibit linear epitopes located at the external faces of the protein and thereby being exposed to solvent. However, after submitting hazelnut samples to autoclaving (121ºC or 138ºC, 15 or 30 min), the allergenicity of Cor a 9 seemed to be affected by this procedure since no comparable size band corresponding to this protein was visible by SDS-PAGE analysis. This finding suggests that the allergenicity of Cor a 9 is predominantly related to structural conformation and not to linear epitopes (López et al., 2012). In the same study, other food processing procedures were tested, namely the application of high pressure processing (300 to 600 Mba) to hazelnut samples, which did not induce any effects on the IgE binding capacity of Cor a 9 in the test population. By means of SDS-PAGE analysis the protein pattern of Cor a 9 subjected to high pressure processing remained similar to the profile presented by the allergen of raw hazelnuts (López et al., 2012).

Cor a 10 (Luminal binding protein)

Cor a 10 is an airborne allergen present in hazel trees. The nucleotide sequence contains 2,007 bp of open reading frame encoding a protein of 668 aa, with a molecular mass of 73.5 kDa and acidic properties (pI 4.8) (Table 1). The deduced primary sequence exhibits 14 potential phosphorylation sites, namely one for tyrosine kinase, five for protein kinase C and eight for casein kinase II. A BLAST search evidences that Cor a 10 has high sequence identity with other luminal binding proteins. As example, protein Cor a 10 showed 90% of sequence identity with BLP-4 and BLP-5 from *Nicotiana tabacum* (CAA42659.1, CAA42660.1), with the heat shock 70 kDa protein 12 from *Arabidopsis thaliana* (AAB86942.1) and with the endoplasmic reticulum HSC70-cognate binding protein from *Glycine max* (BAA12348.1) (Gruehen et al., 2003; NCBI, 2013). Cor a 10 belongs to the 70 kDa heat shock proteins (Hsp70) that comprise a family of molecular chaperones ubiquitously expressed in nature. The Hsp70 proteins can be found in nearly all living organisms, presenting similar structures and functions, namely participating in protein biogenesis, transport and degradation mechanisms (Morano, 2007). As member of the Hsp70 family, Cor a 10 is described as stress-related protein with the capacity to bind other peptides and thus assisting the conformational protein-folding events. Cor a 10 interaction is adenosine 5'-triphosphate (ATP) dependent and the functional protein structure is contained within the N-terminal ATPase region of 45 kDa (1-435), being followed by a 141 aa binding domain (436-577) and a C-terminal regulatory site of 75 aa (578-654) (Gruehen et al., 2003). Physicochemical properties of Cor a 10 allergen are according to those attributed to other known allergens, namely polymorphism, acidic isoelectric point, IgE-binding capacity and cross-reactivity (Gruehen et al., 2003; Stanley and Bannon, 1999). The high sequence identity of Cor a 10 with other Hsp70 chaperones seems to help explaining the ubiquitous expression of chaperone homologues and their relation to protein synthesis rates in different tissues. Pollen, fruits and nuts are naturally resistant to adverse environmental conditions, thus presenting high amounts of Hsp70 stress proteins. This evidence suggests that pollen-sensitised patients with specific IgE against Cor a 10 could developed allergy towards the consumption of plant foods. Molecular chaperons such as Cor a 10 have a key role in the expression and structural integrity of several proteins, which might contribute to maintain the molecular integrity of allergens in different plant tissues. In addition, Cor a 10 possesses IgE-binding activity, which transform this protein in a potential pan-allergen (Gruehen et al., 2003).

Cor a 11 (7S Globulin - Vicilin)

The vicilin-like proteins belong to the cupin superfamily. Like the legumins, the vicilins also have two conserved domains that classify them as bicupins. Mature 7S globulins are trimeric proteins ranging from 150 to 190 kDa, with subunits exhibiting a molecular weight of 40-80 kDa each (Breiteneder and Radauer, 2004). Both 11S and 7S globulins show similar conformational structures, although the primary sequence of vicilins does not contain residues of cysteine (Shewry et al., 1995). The structural integrity of vicilins is guaranteed by non-covalent hydrophobic interactions, hydrogen bonds and van der Waals interactions, while in legumins the tri-dimensional structure is ensured by disulphide bonds. With two structurally equivalent N- and C-terminal domains, legumins and vicilins comprise the cupin beta-barrel conformation, though the vicilins are usually glycosylated proteins with one or two N-linked glycosylation sites in the C-terminal domain (Mills et al., 2002). Classified as vicilins, Cor a 11 has been identified as one of the allergenic groups of proteins in hazelnut. This allergen is encoded by the *Corylus avellana* 48 kDa glycoprotein precursor with 1347 bp and evidences a primary sequence containing 448 aa (Table 1). The mature Cor a 11 has 401 aa with two potential glycosylation sites (Asn^{38} and Asn^{254}) and a signal (leader) peptide of 47 aa (Lauer et al., 2004). This protein reveals high sequence identity with several other plant allergens, namely 67% with Ses i 3 (sesame, AAK15089.1), 64% with Ara h 1 (peanut, AAB00861.1), 52% with Ana o 1 (cashew nut, AAM73730.2), 47% with Jug r 2 (walnut, AAF18269.1) and 46% with 7S vicilin (pecan nut, ABV49593.1) (NCBI, 2013). Additionally, two IgE-binding regions of allergens Ara h 1 and Ana o 1 were compared with the corresponding sequences of other 7S globulins. From this assessment, one IgE-binding epitope of Ara h 1 evidenced approximately 67% of similarity with Cor a 11, and the other IgE-binding epitope evidenced 45% identity with this vicilin (4 out of 5 critical amino acids from IgE of Ara h 1 were identical in Cor a 11). The high degree of similarity between Cor a 11 and Ara h 1 partial sequences seems to suggest they are potential epitopes of Cor a 11 allergen (Lauer et al., 2004).

In general, almost all globulin storage proteins share high predisposition to form large thermally induced aggregates, with propensity to form stable gels and act as emulsifiers, thus their widespread application in food industry (Mills et al., 2007b). The vicilins are considered thermostable proteins with major thermal transition around 70-75ºC, whereas the legumins unfold at higher temperatures (>94ºC) as determined by differential scanning calorimetry, the precise values ranging between plant species, protein concentration and ionic force (Mills et al., 2002; Mills et al., 2007b). When submitted to high temperatures, proteins can suffer conformational disruptions and covalent modifications with special emphasis for those involved in glycation processes or Maillard rearrangements (Mills et al., 2002). Recently, Iwan et al. (2011) evaluated the immunoreactivity and degranulation capacity of Cor a 11 using different temperatures for glycation (37ºC, 60ºC and 145ºC), which resulted in three types of Maillard reaction products. Glycation at 37ºC during 7 days did not allow the formation of coloured products, suggesting that the susceptibility of Cor a 11 for pepsin hydrolysis or its capacity to bind human IgE were not affected. Maillard products from glycation at 60ºC for 3 days and 145ºC for 20 min resulted in decreased hydrolysis by pepsin, indicating that the protein suffers tri-dimensional alterations in the presence of glucose. The physicochemical properties of Cor a 11 were affected after heat treatment at 60ºC and at 145ºC in the presence of glucose. In those conditions, reduced or even no reactivity could be observed between Cor a 11 and IgG/IgE (Iwan et al., 2011). Hazelnut processing using wet heat treatment (121ºC or 138ºC, for 15 or 30 min) also enables to confirm the existence of structure arrangement for the NAG group attached to Asn^{301} residue, as the initial site for glycosylation of the protein by the presence of a sugar (López et al., 2012). Glycation of Cor a 11 suggested a decrease of allergenicity (López et al., 2012), which is in good agreement with the previous study (Iwan et al., 2011). Like for other allergens (Cor a 1, Cor a 8 and Cor a 9), high pressure processing (300-600 Mba) of hazelnuts did not affect the IgE-binding capacity of Cor a 11 allergens. After high pressure processing, the Cor a 11 profile was identical to the protein extracted from raw hazelnuts (López et al., 2012).

Cor a 12 and Cor a 13 (Oleosins)

Oleosins are an intriguing new set of proteins that have been classified as novel allergens in peanut and sesame seeds (Leduc et al., 2006; Pons et al., 2002). The biological functions of these proteins are mainly centred in stabilising lipid bodies (oil bodies), by means of preventing their coalescence during the desiccation of seeds. They possibly interact with the lipidic fraction (lipids and phospholipids) of oil bodies. Oleosins are composed by three distinct domains: a highly conserved hydrophobic central domain of approximately 70 aa (predominantly rich in aliphatic amino acids) flanked by N- and C-terminal domains presenting more hydrophilic affinity and less conserved sequences (Hauser et al., 2008). The C-terminal flanking sequence has an amphipathic alpha-helix that is conserved in several oleosins (Tzen et al., 1992). The central core of these proteins is constituted by one of the longest hydrophobic domains with natural occurrence (Napier et al., 1996). Oil bodies contain triacylglycerols that represent the source of energy for seed germination and growth, thus large amounts of oleosins are required (Akkerdaas et al., 2006).

The allergenic properties of oleosins are not well defined, however their privileged location in oil bodies prevent their detection and identification in nuts and seeds, since mostly diagnostic tools use defatted material. In hazelnut, two representative allergens, Cor a 12 and Cor a 13, have been classified as oleosins. These proteins composed by primary structures of 159 aa and 140 aa, molecular sizes of 16.7 kDa and 14.7 kDa, and presenting basic properties (pI of 10.5 and 10.0) result from the expression of two nucleotide sequences containing 480 bp and 423 bp, respectively, for Cor a 12 and Cor a 13 (Table 1) (Akkerdaas et al., 2006; NCBI, 2013). The sequence identity between those oleosins is only 36% however both present high homology with other oleosins from different plant sources (Akkerdaas et al., 2006). Cor a 12 presented the highest degree of similarity with oleosins classified as allergens such is the case of sesame (50% identity) and peanut (48% identity). Cor a 13 also evidenced a high degree of homology with oleosins from almond (73% identity) and maize (55% identity), however until now, none of those have been related to food allergy. The assembly of oleosins with lipid fraction may be determined to prevent the protection of this allergen towards the rapid proteolysis in the gastrointestinal tract (Akkerdaas et al., 2006). To verify these data, further studies about this topic should be pursuit for better evaluation of the effect of food processing, namely thermal treatment, on the allergenicity of these oleosins.

Cor a 14 (2S Albumins)

Belonging to the prolamin superfamily, hazelnut allergen Cor a 14 is included in the 2S albumins, which along with the globulins comprise the major group of seed storage proteins of dicotyledonous plants (Shewry et al., 1995). The 2S albumins are water soluble proteins at low salt concentrations, presenting a high content of arginine, glutamine, asparagine and cysteine residues. They are small globular proteins that are subjected to sequence modifications after their synthesis. Most of these proteins are cleaved into a large and small subunit (heterodimers) held together by conserved inter-chain disulphide bonds. Like the LTP, the 2S albumins also contain eight cysteine residues that ensure four disulphide bonds. The amino acid composition of the 2S albumins, their high abundance in seed cells and their mobilisation during germination suggest that these proteins act as important nitrogen and sulphur donors (Breiteneder and Ebner, 2000; Hauser et al., 2008). Additional functions such as antifungal properties have also been attributed to some 2S albumins.

The nucleotide sequence containing 633 bp expresses a protein defined as Cor a 14 with a primary structure of 147 aa and a molecular size of 17.1 kDa (ALLERGEN, 2013;

NCBI, 2013). Recombinant 2S albumin from hazelnut has already been cloned from a nucleotide sequence of 441 bp, which allowed encoding a similar 147 aa polypeptide (Garino et al., 2010). This primary sequence comprises a signal peptide of 22 aa, a linker peptide of 20 aa and a mature protein composed of 105 aa. The native protein is predicted to have a molecular size of 12.6 kDa, after post-translational clipping of a N-terminal and internal peptide as described for other 2S albumins. Recombinant and native 2S albumins exhibited similar IgE-activity, suggesting that the availability of these recombinant proteins might help establishing the importance of Cor a 14 regarding hazelnut allergy (Garino et al., 2010). A Blast search evidenced the high sequence identity of Cor a 14 with other allergenic 2S albumins from different tree nuts, namely 63% of similarity with Jug r 1 from walnut (AAB41308.1) and 62% with Car i 1 from pecan nut (AAO32314.1) (NCBI, 2013). These evidences implicate possible cross-reactivity between Cor a 14 and other allergenic 2S albumins from different plant species. Regarding the allergenicity of 2S albumins, not only conformational epitopes, but also shared linear epitopes are apparently related to cross-reactivity phenomena among these proteins (Moreno and Clemente, 2008).

The secondary organisation of 2S albumins, their compact and rigid structure dominated by a well conserved skeleton of cysteine residues are probably responsible for their high stability to the harsh conditions of the gastrointestinal tract (Moreno and Clemente, 2008), thus preserving their allergenic activity. When compared to the nsLTP, the 2S albumins present higher resistance to thermal processing, maintaining their original folding at temperatures up to 90ºC (Mills et al., 2007b).

Cor a TLP (Thaumatin like protein)

The thaumatins are included in the PR-5 group of the pathogenesis-related proteins of the defence system, mainly involved in antifungal activity (Ebner et al., 2001). These proteins evidence a structure containing 16 cysteine residues linked to form eight disulphide bridges, probably contributing to their high resistance to proteases and pH- or heat-induced denaturation (Breiteneder, 2004). Normally, these proteins are divided in three groups according to their biological role that could include responses to pathogen infection, fungal infection or osmotic stress. Cor a TLP was very recently identified as an allergen present in hazelnut (Palacín et al., 2012), although it has not yet been included in the WHO-IUIS list of allergens (ALLERGEN, 2013). Palacín et al. (2012) purified sixteen different TLP in which comprised the hazelnut TLP with the NCBI accession number P83336. However, this accession number identifies a protein with 212 aa as the thaumatin-like protein 1b expressed from a nucleotide sequence with 696 bp of the *Malus domestica* organism. With the available data from this study, it is not possible to state other relevant information about the biochemical proprieties of hazelnut TLP (Palacín et al., 2012), rather than the recognition of these proteins by less than 10% of the tested population that included several patients from seven regions of Spain. The lack of more reliable information suggests that further research work is still needed to correctly identify these proteins. The designation of Cor a TLP can be found in Allergome database, which also categorises their presence in seed tissues, being by ingestion the natural route of exposure to this food allergen.

CLINICAL RELEVANCE OF HAZELNUT ALLERGY

Food allergens are defined as natural food components (proteins/glycoproteins) that are recognised by the immune system and can elicit immunologic reactions in sensitised individuals, resulting in characteristic symptoms (Boyce et al., 2010). A number of specific clinical syndromes may occur as a result of food allergy, which are classified on the basis of inter-related immunologic causes and the organ or system(s) affected (Boyce et al., 2010; Sicherer and Sampson, 2006). Those disorders can vary in intensity, targeting one or more organs/systems, simultaneously. Clinical manifestations of food allergy can include cutaneous reactions (dermatitis, urticarial, angioedema), gastrointestinal disorders (oral allergy syndrome - OAS, eosinophilic gastroenteritis and esophagitis, immediate gastrointestinal hypersensitivity), respiratory syndromes and anaphylaxis (Boyce et al., 2010).

Common clinical symptoms related to hazelnut allergy are often described as mild to potentially life-threatening (anaphylaxis), according to the severity of the elicit response. Allergy to hazelnut is especially frequent in individuals presenting respiratory disorders associated with allergy to pollens from birch, hazel or alder (Ortolani et al., 2000). This fact is linked to the high homology among the allergenic PR-10 proteins, which are known to be responsible for the wide frequency of cross-sensitisation to multiple PR-10 proteins from different fruits, seeds, pollens and nuts. In the northern Europe, most cases of fruit or nut allergy seem to be connected with birch pollinosis, while in southern Europe non-pollen related allergens play an important role in hazelnut allergy, suggesting the existence of different patterns of sensitisation (Akkerdaas et al., 2000; Hirschwehr et al., 1992; Schocker et al., 2000). Belonging to the PR-10 proteins, Cor a 1 are classified both as inhalant and food allergens, being also regarded as major allergens since more than 50% of the allergic patients are skin test reactant to this allergen (Chapman, 2008). In the majority of the cases, clinical manifestations associated with this class of proteins are typically mild and frequently exclusively related to OAS. These facts were demonstrated in a recent study conducted in a birch-endemic region, where 97% of the tested population with OAS were sensitised to Cor a 1.04 and Cor a 1.0101, probably as a result of cross-reactivity with Bet v 1. However, the same study also reported that approximately 24% of preschool children and 50% of school-age children and adults with severe systemic reactions were sensitised to Cor a 1.04 or Cor a 1.0101, thus evidencing the importance of this group of allergens (De Knop et al., 2011).

Classified as pan-allergens due to their widespread distribution throughout nature, Cor a 2 (profilin) and Cor a 8 (nsLTP) are regarded as important allergens in hazelnut, although with very different clinical profiles. Profilins are generally considered as minor (less than 20% of positive skin test responses) (Chapman, 2008), but rather highly relevant allergens such as the case of Cor a 2. The most common route of sensitisation to this allergen is by inhalation and not ingestion since these proteins are greatly affected by heat processing and gastric digestion (García and Lizaro, 2011). The clinical symptoms related to profilins are considered mild and mainly restricted to the oral cavity (OAS), as the result of the ingestion of raw foods. Since profilins can be virtually found in almost all tissues, namely pollen and nuts/seeds, the risk of multiple sensitisation to different pollens and fruits is probabilistically elevated (Hauser et al., 2010). The clinical relevance of profilin sensitisation is still a matter of discussion, as clinical studies seem to suggest that patients displaying profilin-specific IgE antibodies are often asymptomatic or at risk of evolving multiple pollen-related food allergies (Costa et al., 2012a). Moreover, other studies advocate that profilins can be considered as food allergens with clinical relevance in specific food-allergic patients (Asero et al., 2003; Asero et al., 2008), evidencing that further research is obviously still needed. While profilins are considered as minor allergens, the classification of major allergens can be attributed to some nsLTP. In general, the nsLTP are major cross-reactive allergens present in the majority of the plant-derived foods as well as in pollen of diverse plants, nevertheless, the route of sensitisation to these proteins is presumably dependent on geographical differences. The clinical symptoms associated with nsLTP are normally classified as severe immunological responses (Hansen et al., 2009; Hauser et al., 2010).

As other allergenic nsLTP, Cor a 8 have also been reported to induce severe anaphylactic reactions in seven out of 65 patients with allergy to hazelnut (Pastorello et al., 2002). In another study performed in a birch-endemic area, regarding hazelnut allergic patients with systemic reactions, sensitisation to Cor a 8 allergens was observed in 12% of preschool children, 17% of school-age children and 6.7% of individuals over 18-years old. In patients with mild responses (OAS) or in birch pollen allergic individuals without hazelnut allergy, no sensitisation to this allergen could be perceived (De Knop et al., 2011). Recently, it was evidenced that Pru p 3 allergens from peach, which share 59% of sequence identity with Cor a 8, are estimated to function as a primary sensitizer to nsLTP from a large amplitude of unrelated plant-derived foods, including to Cor a 8 (Asero, 2011;

Hartz et al., 2010; Schulten et al., 2011b). Additionally, the level of IgE to peach LTP is regarded as the key issue associated with cross-reactivity and subsequent clinical allergy to foods from different botanical origin (Asero, 2011). Although the classification of major allergen has not yet been attributed to Cor a 8 as for other allergenic nsLTP, the severity of the reactions triggered by these type of proteins cannot be underestimated. The pattern of IgE sensitisation to hazelnut is probably affected by geographical differences, once in the northern Europe the rate of sensitisation to hazelnut is more frequently attributed to Cor a 1 allergens (specifically Cor a 1.04), whereas in the Mediterranean prevailed the IgE towards Cor a 8 (Hansen et al., 2009).

Like the nsLTP and as an important member of the prolamin superfamily, the 2S albumin Cor a 14 was suggested to be connected with moderate to severe hazelnut allergy (Garino et al., 2010; Pastorello et al., 2002). Although the WHO/IUIS identifies Cor a 14 with an IgE-binding prevalence of about 33% (ALLERGEN, 2013), suggesting its classification as minor allergen, relevant information about clinical manifestations and pattern of sensitisation to this allergen remain scarce (Ebo et al., 2012).

Proteins from the cupin superfamily, with biological functions mainly related to nutrient storage, are classified as major components in nuts/seeds. Due to their high abundance in those tissues, the proteins with allergenic properties are frequently considered major allergens in foods. Cor a 9 and Cor a 11, that are 11S legumin- and 7S vicilin-like proteins, respectively, are associated with severe hazelnut allergy. The exact route of sensitisation to Cor a 9 is still not very well defined, but the clinical symptoms regarding this allergen are rather important, since systemic and potentially life-threatening allergic reactions are normally attributed to it (Beyer et al., 2002; Ebo et al., 2012). This fact was demonstrated by Beyer et al. (2002) that reported systemic reactions in 86% of the test population (predominantly children) and by Hansen et al. (2009) that found sensitisation to Cor a 9 in four out of the seven patients with clear severe allergy to hazelnut. De Knop et al. (2011) also reported that in a birch-endemic region the majority of the hazelnut allergic children (65% of preschool children and 50% of school-age children) revealed systemic reactions upon the consumption of processed hazelnut, mostly being sensitised by Cor a 9 with no relation to birch pollen allergy. The sensitisation to Cor a 9 seems to demonstrate a distinct clinical pattern and age-related distribution (Ebo et al., 2012), evidencing that it can occur in very young children prior to pollen sensitisation or allergy, independently from cross-reactivity with other homologues in legumes (soy and peanut) (Verweij et al., 2011). In the same context, a recent study performed among hazelnut-allergic Dutch adults and children with objective symptoms evidenced highly specific sensitisation to Cor a 9 and Cor a 14, whereas sensitisation to Cor a 8 was rare. Although still needing more supporting evidences, this study suggests Cor a 9 and Cor a 14 as possible markers for the evaluation of a more severe hazelnut allergic phenotype (Masthoff et al., 2013).

Regarding Cor a 11 protein, the sensitisation to this allergen has been reported in both hazelnut allergic patients presenting mild immunological responses, mainly related to OAS (Lauer et al., 2004) or experiencing severe systemic reactions (Ebo et al., 2012). Like for Cor a 9, the sensitisation to Cor a 11 was more prominent in children with systemic responses than in adults with the same clinical symptoms (Ebo et al., 2012). The route of sensitisation to this allergen, as for the Cor a 9, is also not defined though it seems to follow the same pattern of the latter. The classification of major allergen has also been advanced for Cor a 11 (Cucu et al., 2012a; Rigby et al., 2008), nonetheless this designation seems to be overestimated since in most of the reports the percentage of positive reactions to Cor a 11 is lower than 50% (Ebo et al., 2012; Lauer et al., 2004; Verweij et al., 2012). Hence, the designation of minor allergen should be more adequate to classify Cor a 11.

The clinical relevance and the pattern of sensitisation of the oleosins Cor a 12 and Cor a 13 are not yet defined. As described for other oleosins, namely from peanut (Ara h 10 and Ara h 11) and sesame (Ses i 4 and Ses i 5) (Leduc et al., 2006; Pons et al., 2002), the oleosins from hazelnut also reveal IgE-binding activity (Akkerdaas et al., 2006). Recent data about the allergenicity of Cor a 12 and Cor a 13 estimated an IgE prevalence of 63% corresponding to about 118 individuals with positive immunological responses in a total of 185 patients with hazelnut and/or peanut allergies (ALLERGEN, 2013). Although the IgE-binding capacity seems to be rather elevated, the purified fraction of oleosins from hazelnut also contained an unidentified 27 kDa protein and the 11S globulin (ALLERGEN, 2013), which might affect the estimative of the IgE activity of these oleosins. Taking into consideration the high percentage of positive reactants to Cor a 12 and Cor a 13, it is possible to suggest that these proteins could be classified as major allergens.

Presently, relevant information correlating the different groups of allergenic proteins in hazelnut and their respective symptoms/clinical relevance as well as sensitisation patterns, is slowly becoming available. Still further research studies based on multidisciplinary teams (clinicians, immunologists, researchers) must be pursuit in order to enable better tools for the management of food allergies, namely hazelnut allergy.

STRATEGIES FOR DETECTING HAZELNUT ALLERGENS IN FOODS

The need for adequate methodology to evaluate the presence of allergenic ingredients and hidden allergens in foods has been, for a long time, a source of extensive discussion and contradictory opinions among the researchers. The lack of harmonisation regarding the most suitable methodology to verify labelling compliance and the absence of available testing/reference materials contribute to the generalised controversy among researchers and represent key issues in the management of food allergens. While in the opinion of some, the direct monitoring of the offending proteins should always be addressed, others defend that alternative methodologies via the indirect assessment of allergenic foods (DNA) can also be considered valuable tools. Presently, there are a great number of methods, either based on proteins or DNA, available for the detection of hazelnut as an ingredient or a potential hidden component in foods.

Protein-based methods

The most representative and widely used techniques for allergen monitoring in foods are the immunochemical assays such as enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD), dipstick tests, immunoblotting and biosensors. All these assays are based on the same principle, which consists on the interaction between an antibody (Ig) and an antigen (protein). Thus, protein-based techniques allow detecting the offending food directly, either targeting the allergenic protein itself or other protein marker(s).

ELISA systems

ELISA is considered the most largely applied type of immunoassay for the detection of allergenic ingredients/non-ingredients in foods with the advantage of providing quantitative information. In general, the most common format of ELISA is the sandwich type, but these immunoassays can also present other forms that are included in two groups: the competitive and the non-competitive assays. For the detection and quantification of hazelnut in foods, several ELISA kits are commercially available and listed in Table 2. The application of ELISA kits to food analysis presents the advantages of rapid performance and versatility, high reproducibility and reliable detection of trace amounts of hazelnut proteins in foods down to 0.3 mg/kg (Table 2). These limits of detection (LOD) are considered low and very adequate to trace hazelnut in foods, once it is known that minute amounts of this nut could induce allergic reactions in sensitised individuals.

Although with very well established advantages, is important to mention that the performance of the ELISA kits can be affected by the composition of foods (e.g. matrix effects) and by the effect of food processing (e.g. heat treatments, formation of Maillard products, fermentation, partial hydrolysis) (Cucu et al., 2011; Cucu et al., 2013; Garber and Perry, 2010; Pele et al., 2007; Platteau et al., 2011a; Platteau et al., 2012; Roder et al., 2009).

Table 2 Commercial ELISA, LFD and real-time PCR kits for the detection and quantification of hazelnut allergens

Commercial kits/Brand	Assay type	Cross-reactivity	LOD	LOQ	Estimated time to perform assay
Lateral Flow Hazelnut (R-Biopharm AG Darmstadt, Germany)	LFD	Walnut: 0.1%, Pumpkin seed: 0.01 %	1.5 mg/kg	2.5 mg/kg	~10 min (sample preparation)
Reveal 3-D for hazelnut (NEOGEN, Michigan, USA)	LFD	No available information about the specificity	5-10 mg/kg	-	~5 min
AgraStrip Hazelnut (Romer Labs Division Holding GmbH, Austria)	LFD	No available information about the specificity	-	-	-
Ridascreen Fast Hazelnut (R-Biopharm AG Darmstadt, Germany)	Specific antibody against hazelnut proteins, sandwich ELISA	No apparent cross-reactivity with 31 plant-derived foods and 2 animal-derived foods	1.5 mg/kg	2.5 mg/kg	~40 min (10 min sample preparation, 30 min ELISA)
AgraQuant Hazelnut Assay (Romer Labs Division Holding GmbH, Austria)	Quantitative - Sandwich ELISA	No cross-reactivity with 30 plant-derived foods	0.3 mg/kg	1 mg/kg	~60 min
DIA hazelnut (Diagnostic Automation, Inc., California, USA)	Quantitative - Sandwich ELISA	No cross-reactivity with 31 plant-derived foods	0.33 mg/kg	1 mg/kg	~50 min (applied to extracted sample)
ELISA Systems Hazelnut (Queensland, Australia)	ELISA	ELISA Systems			~35 min (applied to extracted sample)
Veratox for hazelnut allergen (NEOGEN, Michigan, USA)	Quantitative - Sandwich ELISA	No available information about the specificity	2.5 mg/kg	2.5 mg/kg	~30 min
SureFood Allergen Hazelnut (R-Biopharm AG Darmstadt, Germany)	Real-time PCR (qualitative)	No available information about the specificity	≤5 DNA copies, ≤0.4 mg/kg	-	~30 min (applied to extracted sample)
SureFood Allergen Quant Hazelnut (R-Biopharm AG Darmstadt, Germany)	Real-time PCR using the laboratory reference material SureFood Quantard Allergen 40 (quantitative)	No available information about the specificity	0.4 mg/kg	0.4 mg/kg	~40 min (applied to extracted sample)

To address the referred issues, some studies have been conducted aiming at evaluating the performance of commercial kits from different brands to detect hazelnut traces in processed foods (Cucu et al., 2011; Garber and Perry, 2010). For the same set of samples spiked with hazelnut (chocolate, baked muffins and cooked oatmeal), three different brands of commercial ELISA kits were tested by Garber and Perry (2010), reporting that each kit performed very distinctly. When applied to processed foods such as baked muffins, the three kits revealed poor recoveries and dynamic ranges, which seem to indicate that the heat treatment led to possible alterations on the structural conformation of the target proteins. The modification of the natural folding of native protein structures is quite frequent with processing at elevated temperatures. The same results regarding the negative effect of heat treatment towards the detection of hazelnut proteins by different commercial ELISA kits were also reported by Cucu et al. (2011).

Proteins are also susceptible of suffering severe chemical modifications as a consequence of Maillard reactions, thus resulting in a distinct tri-dimensional conformation of the native protein. Most of the available ELISA kits are probably developed upon antibodies that were raised against raw hazelnuts. This fact can conduct to a reduced capacity of the antibodies to recognise proteins modified by Maillard reactions and, consequently, provide erratic results when analysing processed foods (Cucu et al., 2011). While the native structure of proteins is affected by heat treatment that destroys conformational epitopes, linear epitopes are mainly altered by interactions between lipids or carbohydrates and proteins through partial hydrolysis (Cucu et al., 2013). This procedure can be used in food processing, contributing to a general decrease of the allergenicity. Different ELISA kits were tested with extracts of hazelnut proteins submitted to partial hydrolysis. In general, the results evidenced a reduction in the recognition of the hydrolysed proteins by the antibodies, though one of the tested kits exhibited good performance towards the target proteins (Cucu et al., 2013). All these facts seem to emphasise that the choice of an ELISA kit is highly dependent on the purpose of the analysis, therefore reliant on the type of food matrices and on the kind of processing the samples were submitted to (Cucu et al., 2013).

In addition to the commercial kits, in the last decade, other ELISA tests have been proposed regarding the detection and quantification of hazelnut in foods (Table 3). Most of the developed ELISA employed polyclonal antibodies (IgG from rabbit and sheep or IgY from hen’s eggs) that were generally raised against raw and/or roasted hazelnut protein extracts or purified allergen fractions (e.g. corylin). Table 3 assembles all the proposed ELISA systems reported in the literature. Those assays presented elevated performance with LOD ranging from 0.1-3 mg/kg of hazelnut protein in foods, being applied to a wide variety of food samples, namely cookies, biscuits, chocolates, breakfast cereals, ice-cream, cereal bars and olive oils. The specificity of ELISA using polyclonal antibodies is usually good, but in some cases some cross-reactivity can be observed with other nuts or legumes. The ELISA protocols employing anti-hazelnut monoclonal antibodies are commonly less prone to cross-react with other species. However, since monoclonal antibodies have poorer affinities than the corresponding polyclonal antibodies, the assays using monoclonal antibodies are frequently less sensitive (Table 3). In sandwich ELISA format, mono- and polyclonal antibodies could be combined aiming at developing a more sensitive and specific assay.

Lateral Flow Devices and Immunoblotting

Lateral flow devices or dipstick assays are another type of immunochemical tests applied to the detection of hazelnut in processed products. In food industry, these assays are commonly used for rapid screening of possible cross-contaminations in the production lines. This type of tests provides qualitative or semi-quantitative information that can be easily interpreted visually. In addition, LFD present the advantage of being rapid and simple to perform, without the need of using specialised equipment or personnel (Schubert-Ullrich et al., 2009). Like the ELISA, there are two types of LFD: the sandwich and the competitive formats. The application of commercial LFD enables detecting down to 1.5 mg/kg of hazelnut proteins in foods in approximately 5-10 min (Table 2), which represents a major benefit for food industry. Using LFD from three distinct brands, Roder et al. (2011) reported the detection of hazelnut with two of the tested LFD in spiked chocolate and cookie dough down to 3.5 mg/kg and 2.6 mg/kg, respectively. As for ELISA kits, the choice of the adequate LFD should be performed carefully, taking in consideration the finality of the test.

Concerning all the advantages, LFD also pose some drawbacks such as the lack of quantitative information and the susceptibility of providing false-negative results (Diaz-Amigo, 2010).

Immunoblotting assays are also used to detect allergens in foods, although it is not considered a suitable method for routine analysis. The application of this technique enables the evaluation of the antibody specificity and the occurrence of cross-reactivity between non-target proteins and the applied antibody. Scheibe et al. (2001) have reported a sensitive protocol to trace hazelnut in chocolate using SDS immunoblot with chemiluminescence detection method with a LOD of 5 mg/kg of hazelnut protein in chocolates. Due to the lack of reliable quantification data, immunoblotting is rather used as confirmatory analytical tool during the development of other immunochemical assays.

Biosensors

The application of biosensors as alternative platforms for the detection of allergens in foods has become one of the most emerging and attractive fields, whose advances and future trends have been recently highlighted by Pilolli et al. (2013). When compared to other protein-based methods such as ELISA, biosensors are regarded as one of the most promising ways to solve some issues concerning simple, fast, reproducible and low cost multitarget detection. In addition to these advantages, biosensors are featured to be of high speed of execution, ease to use and feasible for automation. Considering all the potential attributed to these analytical devices, it is expected that biosensors could be applied at industrial scale for the direct and in real-time monitoring of allergens along a production line (Pilolli et al., 2013).

Table 3 Summary of the protein- based methods for the detection and quantification of hazelnut allergens in foods available in the literature.

Protein-based methods	Antibody/target protein (immunisation)	Cross-reactivity	Sensitivity levels	Application to food matrices	Reference
Sandwich ELISA (Quantitative)	Polyclonal rabbit and sheep antibodies (raised against raw and toasted hazelnut, mainly Corylin fraction	3/39 animal- and plant-derived foods. Cross-reactivity with cashew (2.1 mg/kg), pumpkin seeds (6.4 mg/kg) and walnut (4.6 mg/kg)	10 mg/kg of hazelnut (1 mg/kg of hazelnut protein)	Chocolates, breakfast cereals, bar cereals and cookies (spiked and commercial)	Holzhauser and Vieths (1999)
Sandwich ELISA	Rabbit anti-hazelnut IgG (raised against crude hazelnut protein)	6/27 plant-derived foods. Cross-reactivity for cashew (34 mg/kg), walnut (787 mg/kg), brazil nut and almond (28 mg/kg), pine (10 mg/kg) and peanut (11 mg/kg)	10 mg/kg of hazelnut (1 mg/kg of hazelnut protein)	Cakes, chocolates, cookies (commercial)	Koppelman et al. (1999)
Sandwich enzyme immunoassay (EIA)	Polyclonal hen's egg yolk IgY (raised against hazelnut globulin fraction)	0/4 almond, pecan, walnut and sesame. No cross-reactivity observed.	<1 mg/kg of hazelnut protein	Cakes, ice-cream, chocolate, fruit bars (spiked and commercial)	Blais and Phillippe (2001)
Sandwich ELISA and dipstick immunoassay	Polyclonal rabbit antibody (raised against raw hazelnut - Corylin fraction), polyclonal sheep antibody (raised against native and heated hazelnut Corylin)	5/23 plant-derived foods. Weak cross-reactivity with kidney beans, pine, coconut and mix cereals (wheat, rye, maize, rice, oat and barley). Strong cross-reactivity with walnut.	10 mg/kg of hazelnut (1 mg/kg of hazelnut protein) for both ELISA and dipstick	Chocolates creams, chocolate bars, cereal bars, cookies (commercial)	Stephan et al. (2002)
Competitive ELISA	Polyclonal rabbit IgG (raised against defatted roasted hazelnut protein extracts)	0/39 plant- and animal-derived foods. No cross-reactivity observed at 1 mg/mL protein.	0.25 mg/kg of hazelnut protein	Chocolates (spiked and commercial)	Rejeb et al. (2003)
Multiplex EIA system	Polyclonal hen's egg IgY (raised against defatted raw hazelnut - mainly globulin fraction)	0/2 peanut and brazil nut. No cross-reactivity with peanut or brazil nut at 1 mg/kg of protein.	0.1 to 1 mg/kg of hazelnut protein	Chocolate, ice-cream and cookies (spiked)	Blais et al., (2003)
Indirect competitive ELISA	Polyclonal hen's egg IgY (raised against defatted raw hazelnut)	13/29 plant- and animal-derived foods. Cross-reactivity with chestnut, brazil nut, chickpea, green pea, bean, sunflower, sesame, poppy seed, wheat, corn, oats, barley, yeast ranging from 0.30% to 8.09 %.	10 µg/L of hazelnut protein (estimated as the baseline of the method)	Cookies (spiked)	Drs et al. (2004)
Sandwich ELISA	Polyclonal rabbit IgG (raised against hazelnut pepsin digest and hazelnut not digested extracts)	9/10 plant-derived foods. Cross reactivity with peanut (340 mg/kg), walnut (170 mg/kg), almond (80 mg/kg) and sesame (20 mg/kg). Cross-reactivity with wheat, brazil nut, pine, barley and birch pollen (<10 mg/kg).	1 mg/kg of hazelnut	Chocolate (spiked) Chocolates and cookies (commercial)	Akkerdaas et al. (2004)
Competitive ELISA (multitarget)	Polyclonal rabbit IgG (raised against defatted roasted hazelnut protein extracts)	5/24 plant- and animal-derived foods. Cross-reactivity with almond, cashew, egg and lobster. Weak cross-reactivity for chocolate.	1 mg/kg of hazelnut protein	Milk and dark chocolates (spiked)	Rejeb et al. (2005)
Sandwich ELISA	Monoclonal mouse IgG, polyclonal rabbit IgG (raised against roasted hazelnut extracts)	4/21 plant-derived foods. Cross-reactivity with macadamia (1.1 mg/kg), almond and cashew (1.2 mg/kg) and walnut (12 mg/kg).	0.2-1.2 mg/kg	Dark and milk chocolates, cereals, cookies and ice-cream (spiked and commercial).	Kiening et al. (2005)
Sandwich ELISA	Polyclonal rabbit IgG (raised against native hazelnut - corylin fraction)	1/26 plant- and animal-derived foods. Cross-reactivity with walnut (1.1 mg/kg)	0.1 mg/kg hazelnut protein	Cereals, cookies, dark and milk chocolates (spiked and commercial)	Faeste et al. (2006)

Table 3 (continued) Summary of the protein- based methods for the detection and quantification of hazelnut allergens in foods available in the literature.

Protein-based methods	Antibody/target protein (immunisation)	Cross-reactivity	Sensitivity levels	Application to food matrices	Reference
Sandwich ELISA	Polyclonal hen's egg yolk IgY (raised against Cor a 9 allergen)	4/22 plant-derived foods. Cross-reactivity with pecan (80 mg/kg), coconut and beans (20 mg/kg) and macadamia (17 mg/kg)	0.1 mg/kg of Cor a 9 in food	Cookies (spiked)	Trashin et al. (2011)
Indirect competitive ELISA	Polyclonal hen's egg yolk IgY (raised against modified hazelnut proteins - raw and roasted hazelnut protein extract + glucose + sunflower oil)	2/22 plant- and animal-derived foods. Cross-reactivity with walnut (8.2%) and pecan (5.9%)	3 mg/kg of hazelnut protein in blank cookies	Baked cookies (spiked)	Cucu et al. (2012b)
SPR biosensor (optical transducer)	Polyclonal rabbit IgG and hen's egg yolk IgY (raised against hazelnut proteins with molecular weight ranging from 15-30 kDa and predominant band at 18 kDa - Corylin)	Not verified	5 mg/kg of hazelnut	Dark and milk chocolates, ice-creams, bread, biscuits, pasta (commercial)	Yman et al. (2006)
Direct biosensor (optical transducer)	Monoclonal mouse IgG (raised against raw and roasted hazelnut extracts)	0/40 plant- and animal-derived foods. No cross-reactivity observed.	0.08 mg/kg of hazelnut proteins	olive oils (spiked)	Bremer et al. (2009)
SPR biosensor (optical transducer) (multitarget)	Monoclonal mouse IgG (raised against raw and roasted hazelnut extracts)	0/40 plant- and animal-derived foods. No cross-reactivity observed.	1.5 mg/kg and 4.6 mg/kg of hazelnut proteins in cookies and dark chocolates, respectively	Dark chocolates and cookies (commercial)	Rebe Raz et al. (2010)
Electrochemical biosensor (amperometric transducer)	Polyclonal hen's egg yolk IgY (raised against Cor a 9 allergen)	4/22 plant-derived foods. Cross-reactivity with pecan (80 mg/kg), coconut and beans (20 mg/kg) and macadamia (17 mg/kg)	1 mg/kg of Cor a 9 in food	Cookies (spiked)	Trashin et al. (2011)
Immunoblotting	Polyclonal rabbit antibody (raised against hazelnut and almond proteins)	0/3 peanut, cocoa and milk. No cross-reactivity observed.	5 mg/kg of hazelnut proteins	Chocolates (spiked)	Scheibe et al. (2001)
MALDI-TOF/TOF MS	Not applicable/peptides from hazelnut (oleosins and Cor a 9)	Not verified	1% of hazelnut oil (peptides from oleosins and Cor a 9)	Extra-virgin olive oil	Arlorio et al. (2010)
LC-ESI-LIT-MS/MS (multiallergen approach)	Not applicable/peptides from hazelnut (1 Cor a 9 peptide)	Not verified	30 or 35 mg/kg of Cor a 9 according to the MS^2 or MS^3 acquisition mode, respectively	Biscuits (spiked) Cereal mixes, biscuits (commercial)	Bignardi et al. (2010)
LC-MS/MS (multiallergen approach)	Not applicable/peptides from hazelnut (4 Cor a 9 peptides)	Not verified	5 mg/kg of hazelnut	Bread and flour (spiked)	Heick et al. (2011a)
LC-MS/MS (multiallergen approach)	Not applicable/peptides from hazelnut (4 Cor a 9 peptides)	Not verified	5 mg/kg of hazelnut	Bread material (spiked)	Heick et al. (2011b)
LC-MS/MS	Not applicable/8 peptides from hazelnut (1 from Cor a 8, 4 from Cor a 9 and 3 from Cor a 11)	1 out of 4 peptides from Cor a 9 occurs in walnut, pecan, pistachio and cashew. 1 out of 3 peptides from Cor a 11 occurs in walnut and pecan.	Not defined	Not tested in food samples	Ansari et al. (2012)

Biosensors base their principle on the direct recognition of a biological interaction between an antibody and a target protein by a transducer that produces a measurable signal. This interaction can be monitored by different types of transducers (optical, acoustical, amperometrical or potentiometrical), generating a signal that is further processed to give a proportional output to the concentration of a specific target. According to the type of transducer, biosensors can be classified as optical, piezoelectric or electrochemical. The detection of allergenic material in foods (e.g. hazelnut) using biosensor technology has been restricted to a small number of antibody-based applications (Table 3). From those, the optical biosensors are the most commonly used, relying their function on the phenomenon of surface plasmon resonance (SPR) that measures the changes in the refractive index when the antibody bonds to the target protein (Diaz-Amigo, 2010; Pilolli et al., 2013). The employment of an optical biosensor was successfully achieved by Yman et al. (2006) for the detection of hazelnut in diverse food matrices, namely chocolates, ice-creams, bread, pasta and biscuits. For the elaboration of this biosensor, those authors used polyclonal rabbit IgG and hen's egg IgY raised against the corylin fraction, allowing to trace minute amounts of hazelnut until the spiked level of 5 mg/kg. The application of optical biosensors based on the same principle was also successfully reported by Bremer et al. (2009) and Rebe Raz et al. (2010). Both optical biosensors evidenced high specificity for hazelnut detection since the monoclonal IgG from mouse used in those systems did not cross-react with any of the forty tested foods. The biosensor proposed by Bremer et al. (2009) presented a LOD of 0.08 mg/kg of hazelnut proteins in spiked olive oil, with recoveries ranging from 93% to 109% and an assay time of about 4.5 min. Using an optical biosensor based on SPR but with multitarget approach (microarray), Rebe Raz et al. (2010) showed the applicability of this system for the simultaneous detection of several allergenic foods. In the specific case of hazelnut, LOD of 1.5 mg/kg and 4.6 mg/kg of hazelnut proteins in cookies and in dark chocolates, respectively, were attained by those authors, which were also in good agreement with the sensitivities reported for ELISA or LFD systems (Table 2 and Table 3).

Trashin et al. (2011) described the development of an electrochemical biosensor, as an alternative approach to ELISA, using in both methods the same type of polyclonal hen's egg yolk IgY raised against Cor a 9 fraction. The system allowed detecting down to 1 mg/kg of Cor a 9 in spiked cookies, although the antibodies evidenced some cross-reactivity with four (pecan nut, coconut, beans and macadamia) out of the 22 plant-derived food tested. When compared with the ELISA system, the LOD attained with the electrochemical biosensor was approximately ten-fold higher, however with the strong advantage of reducing the analysis time from four to one hour. Therefore, the developed biosensor proved to be adequate for the detection of minute amounts of hazelnut in food samples with possible application for the food industry (Trashin et al., 2011). These findings seem to support the emergent potential of biosensors for the detection of allergenic ingredients in processed foods, especially if displayed in platforms to, simultaneously, targeting multiple allergens.

Mass spectrometry (MS)-based methods

The development of novel strategies for allergen detection, quantification and characterisation is a constant demand. Recently, the application of proteomic methodologies (allergenomics) for the analysis of food allergens has been addressed, especially centred on core technologies such as the MS-based platforms. This technology evidences several advantages because it allows proteins to be quickly analysed with high sensitivity, accuracy, specificity and reproducibility (Picariello et al., 2011). In addition, MS methods can overcome the problems of cross-reactivity phenomena often linked to immunoassays, allowing the unequivocal confirmation of the identity of the tested proteins/peptides (Monaci and Visconti, 2009). Several MS-based methods can be used for the relative and absolute quantification of proteins (e.g. allergens), but all of those rely on one of two approaches. In the first one, the analysis is performed using intact proteins (analyte and reference standards), while in the second approach the target analytes are peptides obtained from protein digestion by proteolytic enzymes (Picariello et al., 2011). The identification of allergens or protein markers by MS technology is commonly performed in "bottom-up" mode that is conducted on the basis of the digestion of proteins by a protease, typically trypsin (Monaci and Visconti, 2009). Prior to mass analysis and data recording, proteolytic fragments are separated by reverse-phase liquid chromatography (LC) (Harrer et al., 2010). Due to the complexity of the proteins, the purification process has to be specifically developed to ensure reliable recognition of the molecule via the generation of a peptide mass fingerprinting. With respect to the detection of proteins/allergens in processed foods, MS methods are effective means of providing reliable insights about any protein/peptide modification or changes in their conformation resulting from food processing (Monaci and Visconti, 2009).

Some applications using MS techniques have been reported for the detection and quantification of hazelnut in foods, being listed in Table 3. Using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), Arlorio et al. (2010) described the identification of two oleosin isoforms and Cor a 9. With this method, those authors were able to detect the addition of 1% of hazelnut oil to extra-virgin olive oils, evidencing that this adulteration might also represent a potential risk for allergic patients (Arlorio et al., 2010). The application of a system based on liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry (LC-ESI-LIT-

MS/MS) was successfully achieved by Bignardi et al. (2010) for the simultaneous detection of five nut allergens: Ana o 2 (cashew), Ara h 3/4 (peanut), Pru 1 (almond), Jug r 4 (walnut) and Cor a 9 (hazelnut). In the specific case of hazelnut, those authors reported sensitivities of 30 mg/kg or 35 mg/kg of Cor a 9 in spiked biscuits and LOQ of 90 mg/kg or 110 mg/kg of Cor a 9 in the same matrix, respectively, using MS^2 or MS^3 acquisition modes. No cross-reactivity assessments with peptide from different plant- or animal-derived foods were described (Bignardi et al., 2010). The development of a multiallergen approach for the simultaneous detection of soy, milk, egg, peanut, walnut, almond and hazelnut using LC-MS/MS was also successfully achieved by Heick et al. (2011a) and Heick et al. (2011b). For the identification of hazelnut, four Cor a 9 peptides were targeted in spiked matrices of bread and flour. The method allowed tracing 5 mg/kg of Cor a 9, both in incurred bread and flour, demonstrating elevated sensitivity of the proposed method for raw and processed food samples. A similar LC-MS/MS system was also reported by Ansari et al. (2012) using eight different peptides from Cor a 8, Cor a 9 and Cor a 11 allergens for the unequivocal identification of hazelnut. Although without reporting the level sensitivity, those authors evaluated the potential application of the selected hazelnut peptides for the identification of this nut in processed foods. One out of four Cor a 9 peptides also occurs in pecan, walnut, pistachio and cashew, and one out of the three Cor a 11 peptides is present in walnut and pecan, which evidenced that similar peptides can be found in different foods. Therefore, the use of single peptides should be avoided for the unambiguous identification of hazelnut from other nuts or foods as demonstrated by Ansari et al. (2012). MS methodologies have been applied only very recently, but with high potentiality in the field of food allergen assessment. Further research using MS platforms is expected shortly.

DNA-based methods

The detection of food allergens by DNA-based methods has been extensively applied in the last decade, despite some criticisms. In opposition to the immunochemical assays that exhibit some relevant disadvantages, the only drawback pointed out to the use of molecular approaches for allergen detection regards their indirect assessment in food matrices. In fact, if any protein can act as a marker for species identification, the DNA encoding an allergenic or a marker protein can also play the same role. The elevated stability of DNA upon thermal treatment, pH alterations or partial hydrolysis, which are processes frequently used in food industry, elect this molecule as a favoured target for allergen evaluation. In addition, the molecular methods can be established in routine analysis and function as confirmatory tools for the identification of allergenic foods.

PCR systems

Polymerase chain reaction (PCR) based techniques are among the most widely exploited approaches for the detection and quantification of hazelnut in foods (Table 4), in addition to the fact of being the DNA approaches for which few commercial kits are available (Table 2). The high specificity is one main advantage attributed to these techniques. This characteristic is well evidenced in Table 4 since, in the majority of the developed methods, no cross-reactivity with other plant or animal species could be observed.

In most of the cases, the selected nucleotide sequences encode allergens (e.g. Cor a 1) rather than other proteins, as potential targets for hazelnut identification. Additionally, since no official reference or testing materials are available for hazelnut evaluation, almost all authors choose to elaborate their own set of reference mixtures for the development of molecular approaches as stated in Table 4. Thus, depending on the aim of the research work, model mixtures for PCR-based systems have been prepared using raw, defatted, toasted, roasted or autoclaved hazelnut in food matrices that are also so diversified as wheat material, cookies, chocolate, pasta, walnut or peanut flour (Table 4). The relative limits of detection and quantification of hazelnut in different food matrices range from 100 mg/kg down to 1 mg/kg (Table 4), which are slightly higher than the LOD reported for protein-based methods (Table 3). Despite this fact, the LOD are in good agreement with the intervals considered ideal for allergen evaluation (Poms et al., 2004). In terms of absolute LOD, hazelnut can be targeted in processed foods down to one DNA copy (Costa et al., 2012b), which is theoretically enough to identify the presence of this ingredient. Unlike proteins that can be affected by several variations at the expression level, the DNA complement in a tissue is usually very stable. Thus, it is possible to establish a consistent correlation between the amount of DNA detected and the amount of allergen-containing tissue (Johnson et al., 2011).

The first studies regarding the detection of hazelnut reported the development of methods based on the application of qualitative PCR systems, being the amplified PCR products evaluated on the basis of their differential migration through agarose gel electrophoresis (Herman et al., 2003; Holzhauser et al., 2000). Other qualitative PCR systems coupled with peptide nucleic acid (PNA) HPLC (Germini et al., 2005) or with PNA-array (Rossi et al., 2006) have also been employed.

Table 4 Summary of the reported molecular based methods for the detection and quantification of hazelnut in foods.

Molecular method	Target gene (NCBI accession number)	Fragment size (bp)	Cross-reactivity	Reference standards or model mixtures (range)	Sensitivity levels	Application to commercial foods	Reference
Qualitative PCR, gel electrophoresis	Cor a 1.04 (AF136945)	182	0/32 plant- and animal-derived foods.	Food matrix spiked with hazelnut (100,000-10 mg/kg)	10 mg/kg	Chocolate bars, cereal bars, cookies	Holzhauser et al. (2000)
PCR-ELISA using specific hybridisation probe	Cor a 1.04 (AF136945)	152	0/35 plant- and animal-derived foods.	Hazelnut genomic DNA serially diluted (100-1 pg) Food matrix spiked with hazelnut (100,000-10 mg/kg)	< 10 mg/kg 2pg	Chocolates, cream desserts, cereals, cookies, biscuits	Holzhauser et al. (2002)
Qualitative PCR, gel electrophoresis	Nad1, mitochondrial (AJ428871)	294	4/33 plant- and animal-derived foods. Cross-reactivity with 4 different hazel species	Hazelnut genomic DNA serially diluted (15,000-30 pg) Chocolates spiked with hazelnut paste (50,000-5 mg/kg)	10 mg/kg 300 pg	No data	Herman et al. (2003)
PCR coupled with HPLC using specific PNA probe	Cor a 1.03 (Z72440)	156	0/14 plant-derived foods.	Hazelnut genomic DNA serially diluted (150,000-1 pg)	5 pg	Chocolates, cereals, snacks	Germini et al. (2005)
PCR-array using specific PNA probe	Cor a 1.03 (Z72440)	156	0/14 plant-derived foods (reported by Germini et al., 2005)	Hazelnut genomic DNA serially diluted (150,000-1 pg)	50 pg	Chocolates, cereals, snacks	Rossi et al. (2006)
Real-time PCR using specific hydrolysis probe	Cor a 1 (Z72440)	82	0/14 plant-derived foods	Hazelnut genomic DNA serially diluted (98,000-49 pg)	100 pg	Creams, chocolates, biscuits, corn flakes	Arlorio et al. (2007)
Real-time PCR with hydrolysis probe	Hsp1(AF021807)	100	0/19 plant- and animal-derived foods	Walnut kernel spiked with hazelnut (100,000-100 mg/kg) Hazelnut genomic DNA serially diluted	100 mg/kg 13 pg	Wafers, chocolates, biscuits, corn flakes	Piknová et al. (2008)
Real-time PCR with SYBR-Green I, melt curve	Cor a 8 (AF329829)	78	0/18 plant-derived foods	Wheat flour spiked with defatted hazelnut (100,000-10 mg/kg) Hazelnut genomic DNA serially diluted (30,000-9.6 pg)	10 mg/kg 9.6 pg	Creams, wafers, dark chocolates, biscuits	D'Andrea et al. (2009)
Duplex real-time PCR with hydrolysis probe	Cor a 1 (Z72440)	109	0/25 plant-derived foods	cookies spiked with sesame /hazelnut (10,000-10 mg/kg) sesame/hazelnut DNA serially diluted (100,000-10 pg/µL)	50 mg/kg 50 pg	Cereals, cookies chocolates, snacks, muesli bars, creams	Schöringhumer et al. (2009)
Ligation-dependent probe amplification (multitarget)	Cor a 1.04 (AF136945)	104	0/48 plant- and animal-derived foods	Chocolate spiked with hazelnut (20-5 mg/kg) Walnut cookies spiked with 1% of 5 nut mix (pecan, hazelnut, cashew, macadamia, walnut (10,000-1 mg/kg)	5 mg/kg in chocolate 100 mg/kg in cookies	Chocolates, yogurts, cookies, spreads	Ehlert et al. (2009)
Tetraplex real-time PCR with hydrolysis probe	Cor a 1 (Z72440)	85	1/44 plant- and animal-derived foods. Cross-reactivity with peach (10%)	Rice cookies spiked with hazelnut (50 and 10 mg/kg)	50 mg/kg	Cookies, cakes, cereal bars, biscuits, snacks	Köppel et al. (2010)

Table 4 (continued) Summary of the reported molecular based methods for the detection and quantification of hazelnut in foods.

Molecular method	Target gene (NCBI accession number)	Fragment size (bp)	Cross-reactivity	Reference standards or model mixtures (range)	Sensitivity levels	Application to commercial foods	Reference
Multiplex real-time PCR with SYBR-GreenER, melt curve	No data	54	No data	Mix containing 6 species with 1%. Mix serially diluted (50-0.5 pg of each species)	5 pg	Biscuits, crackers, chocolates	Pafundo et al. (2010)
Real-time PCR with hydrolysis probe	Cor a 8 (AF329829)	218	No data	Defatted hazelnut in flour/cookies dough/ baked cookies (10,000-1 mg/kg)	100 mg/kg (flour and dough) 1000 mg/kg (baked cookies	No data	Platteau et al. (2011a)
Real-time PCR with hydrolysis probe	Cor a 1 (Z72440) Cor a 8 (AF329829)	101 218	0/29 plant- and animal-derived foods	Hazelnut genomic DNA serially diluted (50,000-0.256 pg)	3.2 pg	No data	Platteau et al. (2011b)
Real-time PCR with SYBR Green I, melt curve	Cor a 1 (AF136945) Cor a 8 (AF329829) Cor a 14 (FJ358504)	105 78 116	0/18 plant-derived foods	Wheat flour spiked with defatted hazelnut (100,000-1 mg/kg) Hazelnut genomic DNA serially diluted (30,000-1.92 pg)	10 mg/kg 9.6 pg	Creams, dark chocolates, wafers, biscuits	D'Andrea et al. (2011)
Multiplex ligation-dependent probe amplification (multitarget)	Cor a 1 (AF136945) and (Z72440)	117	0/27 plant foods	No data	48 pg (105 DNA copies)	No data	Mustorp et al. (2011)
Hexaplex real-time PCR with hydrolysis probe	Cor a 1 (Z72440)	85	1/44 plant- and animal-derived foods. Cross-reactivity with peach (10%)	Sausages spiked with hazelnut (3160-32 mg/kg)	32 mg/kg	Sandwiches, lasagne, spices, chocolates, pasta	Köppel et al. (2012)
Single-tube nested real-time PCR (real-time PCR coupled with nested PCR)	Hsp1 (AF021807)	126 97	0/25 plant-derived foods	Wheat material spiked with hazelnut (100,000-10 mg/kg) Hazelnut genomic DNA serially diluted (50,000-0.5 pg)	50 mg/kg 0.5 pg	Chocolates, breakfast cereal	Costa at al. (2012b)
Real-time PCR with SYBR Green I, melt curve	Cor a 9, (JN674437-JN674440) Cor a 11, (JN674445-JN674448) Cor a 13, (JN674441-JN674444)	101 101 101	0/7 plant-derived foods	Peanut flour spiked with defatted hazelnut (500,000-1 mg/kg) Hazelnut genomic DNA serially diluted	1 mg/kg 2.16 pg	Snacks, biscuits, chocolates	Iniesto et al. (2013)
Real-time PCR with hydrolysis probe	Hsp1 (AF021807)	100	0/19 plant- and animal-derived foods (reported by Piknová et al., 2008)	Model chocolates spiked with hazelnut (100,000-1 mg/kg)	50 mg/kg	No data	Costa et al. (submitted)
Electrochemical DNA-array (genosensor)	Cor a 1.03 (Z72440) Cor a 1.04 (AF136945)	156 233	No data	No data	No data	Biscuits snacks, cereals, chocolates, creams	Bettazzi et al. (2008)
Optical thin-films biochips (multitarget)	Oleosin (AY224599)	67	No data	No data	No data	Bread sticks, biscuits, wafers, cookies, noodles	Wang et al. (2011)
Optical DNA-array (multitarget)	Cor a 1 (Z72440)	109	0/25 plant- and animal-derived foods	No data	1 mg/kg	Cereal bars, cookies chocolates, pasta	Tortajada-Genaro et al. (2012)

Lately, due to the recent advances in high resolution instrumentation and with the arising of more specialised fluorescent DNA-binding dyes, the development of real-time PCR systems aiming at quantifying hazelnut allergens in foods has been preferably applied. Using the classical SYBR Green I or the enhancement of SYBR GreenER, several real-time PCR systems have been described with high specificity and sensitivity to trace hazelnut allergens in foods (D'Andrea et al., 2009; D'Andrea et al., 2011; Iniesto et al., 2013; Pafundo et al., 2010). As alternative and for the unequivocal identification of hazelnut in complex food matrices, other studies have demonstrated the use of specific hydrolysis probes to enhance the specificity of the reaction (Arlorio et al., 2007; Costa at al., 2012b; Costa et al., submitted; Köppel et al., 2010; Köppel et al., 2012; Piknová et al., 2008; Platteau et al., 2011a; Platteau et al., 2011b), based on the complementarity of a third hybridisation oligonucleotide during the amplification.

In the past few years, special emphasis has been devoted to the development of multitarget approaches, namely duplex, tetraplex and hexaplex real-time PCR systems for the simultaneous detection and quantification of several allergenic foods including hazelnut (Köppel et al., 2010; Köppel et al., 2012; Pafundo et al., 2010; Schöringhumer et al., 2009). In the same sense, other developed multitarget systems were proposed based on ligation-dependent probe amplification (LPA). Ehlert et al. (2009) developed a LPA technique for the simultaneous identification of 10 allergenic foods that allowed detecting down to 5 mg/kg of hazelnut in chocolates and 100 mg/kg of hazelnut in walnut cookies, thus evidencing its adequacy for the analysis of processed foods. Still based on a similar approach, Mustorp et al. (2011) demonstrated that only the ligated probes are amplified by PCR, which ensures the high specificity and efficiency of the method. Using the proposed system, those authors were able to detect down to 48 pg of hazelnut DNA (105 DNA copies).

Genosensors and Microarrays

Multiplex methods offer the opportunity of detecting several allergens in a single run with the additional benefits of saving time, reducing reagent costs and decreasing the occurrence of possible cross-contaminations. In the case of food safety agencies and food-processing industries that are subjected to detail examination of their control programmes, these features are of extreme importance for the rapid assessment of allergenic ingredients in processed foods (Tortajada-Genaro et al., 2012). Considering all the benefits of the multiplex analysis, some studies have been conducted aiming at developing microarrays and DNA chips for the simultaneous detection of several allergens in a single assay. Bettazzi et al. (2008) described the application of an electrochemical genosensor platform for the detection of PCR fragments obtained from the cDNA of Cor a

1.04 and Cor a 1.03 isoallergens. The development of a silicon-based optical thin-film biochip was proposed by Wang et al. (2011), which enabled to simultaneously detect eight food allergens, including hazelnut, on the basis of two tetraplex PCR systems. Using a different analytical platform, the digital versatile disk technology (drivers and disks), Tortajada-Genaro et al. (2012) reported the successful application of an optical DNA microarray for the detection of PCR fragments from hazelnut, peanut and soybean in foods. With the proposed method those authors obtained sensitivities of 1 mg/kg of each allergenic target. These findings highlight the elevated potential of this technology for the assessment of multiple allergenic foods with virtual application in the food industry. However, much effort is still required for its full development and to comply with this main goal.

FINAL REMARKS

From the available reports, it is clear that tree nuts are regarded as a common cause of food allergy in Europe, from which hazelnut is responsible from a significant part. Common clinical symptoms related to hazelnut allergy are often described as mild to potentially fatal, being frequently associated with allergy to birch pollen. Presently, ten groups of hazelnut allergens have been identified and characterised, from which relevant information regarding their biological function and clinical significance as well as sensitisation patterns have been advanced. From the identified allergens, the nsLTP Cor a 8, and the seed storage proteins Cor a 9, Cor a 11 and Cor a 14 have been associated to severe allergic reactions.

So far, the only actual means of preventing allergic reactions in sensitised individuals consists mainly on the total avoidance of the offending food. Thus, adequate food labelling plays a crucial role in the safeguard of hazelnut allergic patients' health. In this sense, the need for proficient tools to verify labelling compliance has prompted the development of several protein- and DNA-based methods. In spite of the great advances, no official method is yet available for the detection/quantification of hazelnut in foods. This means that many efforts are still required to accomplish harmonisation regarding the most suitable methodology to detect hazelnut and other food allergens.

Considered an important allergenic food, hazelnut is one of the most well studied nuts. The number of publications addressing issues related to hazelnut allergy is high and it is estimated to increase due to the importance of this topic. Until now, no effective treatments concerning hazelnut allergy are available. More recently, clinical trials using oral immunotherapy have been performed, aiming at inducing desensitisation or even tolerance to certain allergenic foods. Although such interventions are still at an early stage and limited to foods such as milk, egg or peanut, their success could represent a clear improvement in the quality of life of the allergic patients. Similar treatments are expected for hazelnut allergy in the near future.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

Akkerdaas, J., Hafle, S., Aalberse, R., and van Ree, R. (2000). Characterization of non-pollen-related hazelnut allergens. *J. Allergy Clin. Immunol.* **105**:S136.

Akkerdaas, J. H., Schocker, F., Vieths, S., Versteeg, S., Zuidmeer, L., Hefle, S. L., Aalberse, R. C., Richter, K., Ferreira, F., and van Ree, R. (2006). Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library. *Mol. Nutr. Food Res.* **50**:18-23.

Akkerdaas, J. H., Wensing, M., Knulst, A. C., Stephan, O., Hefle, S. L., Aalberse, R. C., and van Ree, R. (2004). A novel approach for the detection of potentially hazardous pepsin stable hazelnut proteins as contaminants in chocolate-based food. *J. Agric. Food Chem.* **52**:7726-7731.

Alasalvar, C., and Shahidi, F. (2008). Tree nuts: composition, phytochemicals, and health effects: an overview. **In**: Tree nuts: composition, phytochemicals, and health effects, pp 1-6. Alasalvar, C., and Shahidi, F., Eds., CRC Press: Boca Raton, FL.

Albillos, S. M., Jin, T., Howard, A., Zhang, Y., Kothary, M. H., and Fu, T.-J. (2008). Purification, crystallization and preliminary X-ray characterization of Prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. *J. Agric. Food Chem.*, **56**:5352-5358.

ALLERGEN, (2013). Official site for the systematic allergen nomenclature. Available at: http://www.allergen.org/ Accessed on March 2013.

ALLERGOME, (2013). Allergome database, the platform for allergen knowledge. Available at: http://www.allergome.org/ Accessed on March 2013.

Ansari, P., Stoppacher, N., and Baumgartner, S. (2012). Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts. *Anal. Bioanal. Chem.* **402**:2607-2615.

Arlorio, M., Cereti, E., Coisson, J. D., Travaglia, F., and Martelli, A. (2007). Detection of hazelnut (*Corylus* spp.) in processed foods using real-time PCR. *Food Control* **18**:140-148.

Arlorio, M., Coisson, J. D., Bordiga, M., Travaglia, F., Garino, C., Zuidmeer, L., Van Ree, R., Giuffrida, M. G., Conti, A., and Martelli, A. (2010). Olive oil adulterated with hazelnut oils: simulation to identify possible risks to allergic consumers. *Food Addit. Contam. Part A-Chem.* **27**:11 - 18.

Asero, R. (2011). Lipid transfer protein cross-reactivity assessed in vivo and in vitro in the office: pros and cons. *J. Invest. Allergol. Clin. Immunol.* **21**:129-136.

Asero, R., Mistrello, G., Roncarolo, D., Amato, S., Zanoni, D., Barocci, F., and Caldironi, G. (2003). Detection of clinical markers of sensitization to profilin in patients allergic to plant-derived foods. *J. Allergy Clin. Immunol.* **112**: 427-432.

Asero, R., Monsalve, R., and Barber, D. (2008). Profilin sensitization detected in the office by skin prick test: a study of prevalence and clinical relevance of profilin as a plant food allergen. *Clin. Exp. Allergy* **38**:033-1037.

Bettazzi, F., Lucarelli, F., Palchetti, I., Berti, F., Marrazza, G., and Mascini, M. (2008). Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. *Anal. Chim. Acta* **614**:93-102.

Beyer, K., Grishina, G., Bardina, L., Grishin, A., and Sampson, H. A. (2002). Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J. Allergy Clin. Immunol.* **110**:517-523.

Bignardi, C., Elviri, L., Penna, A., Careri, M., and Mangia, A. (2010). Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J. Chromatogr. A* **1217**:7579-7585.

Blais, B. W., Gaudreault, M., and Phillippe, L. M. (2003). Multiplex enzyme immunoassay system for the simultaneous detection of multiple allergens in foods. *Food Control* **14**:43-47.

Blais, B. W., and Phillippe, L. (2001). Detection of hazelnut proteins in foods by enzyme immunoassay using egg yolk antibodies. *J. Food Prot.* **64**: 895-898.

Blom, W. M., Vlieg-Boerstra, B. J., Kruizinga, A. G., van der Heide, S., Houben, G. F., and Dubois, A. E. J. (2013). Threshold dose distributions for 5 major allergenic foods in children. *J. Allergy Clin. Immunol.* **131**:172-179.

Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., Plaut, M., Cooper, S. F., Fenton, M. J., and the NIAID-sponsored Expert Panel (2010). Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored Expert Panel. *J. Allergy Clin. Immunol.* **126**:S1-S58.

Breiteneder, H. (2004). Thaumatin-like proteins - a new family of pollen and fruit allergens. *Allergy* **59**:479-481.

Breiteneder, H. (2006). Classifying food allergens. **In**: Detecting allergens in food, Koppelman, S. J., and Hefle, S. L., Eds., CRC Press, Boca Raton, FL.

Breiteneder, H., and Ebner, C. (2000). Molecular and biochemical classification of plant-derived food allergens. *J. Allergy Clin. Immunol.* **106**:27-36.

Breiteneder, H., and Radauer, C. (2004). A classification of plant food allergens. *J. Allergy Clin. Immunol.* 113:821-830.

Bremer, M., Smits, N., and Haasnoot, W. (2009). Biosensor immunoassay for traces of hazelnut protein in olive oil. *Anal. Bioanal. Chem.* **395**:119-126.

Burney, P., Summers, C., Chinn, S., Hooper, R., Van Ree, R., and Lidholm, J. (2010). Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy* **65**:1182-1188.

Chapman, M. D. (2008). Allergen Nomenclature. **In**: Allergens and Allergen Immunotherapy 4th Edition, pp 47-58. Lockey, R. F., and Ledford, D. K., Eds. Informa Healthcare, New York.

Chapman, M. D., Pomés, A., Breiteneder, H., and Ferreira, F. (2007). Nomenclature and structural biology of allergens. *J. Allergy Clin. Immunol.* **119**:414-420.

Chafen, J. J. S., Newberry, S. J., Riedl, M. A., Bravata, D. M., Maglione, M., Suttorp, M. J., Sundaram, V., Paige, N. M., Towfigh, A., Hulley, B. J., and Shekelle, P. G. (2010). Diagnosing and managing common food allergies: A systematic review. *J. Am. Med. Assoc.* **303**:1848-1856.

CODEX STAN 1, (1985). Amended in 1991, 1999, 2001, 2003, 2005, 2008 and 2010 regarding the general standard for the labelling of pre-packaged foods. FAO/WHO Standards. *Off. Codex Stand.* Available at: http://www.codexalimentarius.org/standards/list-of-standards/

Costa, J., Mafra, I., Carrapatoso, I., and Oliveira, M.B.P.P. (2012a). Almond allergens: molecular characterization, detection and clinical relevance. *J. Agric. Food Chem.* **60**:1337-1349.

Costa, J., Mafra, I., Kuchta, T., and Oliveira, M. B. P. P. (2012b). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *J. Agric. Food Chem.* **60**:8103-8110.

Costa, J., Melo, V. S., Santos, C. G. M., Oliveira, B. P. P., and Mafra, I. (submitted). Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols. *Food Chem*.

Crevel, R. W. R., Briggs, D., Hefle, S. L., Knulst, A. C., and Taylor, S. L. (2007). Hazard characterisation in food allergen risk assessment: the application of statistical approaches and the use of clinical data. *Food Chem. Toxicol.* **45**:691-701.

Cucu, T., De Meulenaer, B., and Devreese, B. (2012a). MALDI-based identification of stable hazelnut protein derived tryptic marker peptides. *Food Addit. Contam. Part A-Chem.* **29**:1821-1831.

Cucu, T., Devreese, B., Trashin, S., Kerkaert, B., Rogge, M., and De Meulenaer, B. (2012b). Detection of hazelnut in foods using ELISA: challenges related to the detectability in processed foodstuffs. *J. AOAC Int.* **95**:149-156.

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., and De Meulenaer, B. (2011). ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Addit. Contam. Part A-Chem.* **28**:1-10.

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., and De Meulenaer, B. (2013). Effect of partial hydrolysis on the hazelnut and soybean protein detectability by ELISA. *Food Control* **30**:497-503.

D'Andrea, M., Coisson, J. D., Locatelli, M., Garino, C., Cereti, E., and Arlorio, M. (2011). Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via real-time PCR. *Food Chem.* **124**:1164-1171.

D'Andrea, M., Coisson, J. D., Travaglia, F., Garino, C., and Arlorio, M. (2009). Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (*Corylus avellana* L.) in foods through calibration via plasmid reference standard. *J. Agric. Food Chem.* **57**:11201-11208.

De Knop, K. J., Verweij, M. M., Grimmelikhuijsen, M., Philipse, E., Hagendorens, M. M., Bridts, C. H., De Clerck, L. S., Stevens, W. J., and Ebo, D. G. (2011). Age-related sensitization profiles for hazelnut (*Corylus avellana*) in a birch-endemic region. *Pediatr. Allergy Immunol.* **22**:e139-e149.

Diaz-Amigo, C. (2010). Antibody-based detection methods: from theory to practice. **In**: Molecular biological and immunological techniques and applications for food chemists, pp 223-245. Popping, B., Diaz-Amigo, C. and Hoenicke, K., Eds., John Wiley & Sons, Inc., Hoboken, New Jersey.

Directive 2000/13/EC of 20 March 2000 relating to the labelling, presentation and advertising of foodstuffs. *Off. J. Eur. Comm.* **L109**:29-42.

Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC regarding certain food ingredients. *Off. J. Eur. Union* **L310**:11-14.

Dooper, M. M. B. W., Plassen, C., Holden, L., Moen, L. H., Namork, E., and Egaas, E. (2008). Antibody binding to hazelnut (*Corylus avellana*) proteins: the effects of extraction procedure and hazelnut source. *Food Agric. Immunol.* **19**:229-240.

Drs, E., Baumgartner, S., Bremer, M., Kemmers-Voncken, A., Smits, N., Haasnoot, W., Banks, J., Reece, P., Danks, C., Tomkies, V., Immer, U., Schmitt, K., and Krska, R. (2004). Detection of hidden hazelnut protein in food by IgY-based indirect competitive enzyme-immunoassay. *Anal. Chim. Acta* **520**:223-228.

Dunwell, J. M., Purvis, A., and Khuri, S. (2004). Cupins: the most functionally diverse protein superfamily? *Phytochemistry* **65**:7-17.

EAACI, (2013). Food allergy and anaphylaxis public declaration. European Academy of Allergy and Clinical Immunology (EAACI). Available at: http://www.eaaci.org/attachments/FoodAllergy&AnaphylaxisPublicDeclaration.pdf Accession on March 2013

Ebner, C., Hoffmann-Sommergruber, K., and Breiteneder, H. (2001). Plant food allergens homologous to pathogenesis-related proteins. *Allergy* **56**(Suppl. 67):43-44.

Ebo, D. G., Verweij, M. M., Sabato, V., Hagendorens, M. M., Bridts, C. H., and De Clerck, L. S. (2012). Hazelnut allergy: a multi-faced condition with demographic and geographic characteristics. *Acta Clin. Belg.* **67**:317-321.

Ehlert, A., Demmel, A., Hupfer, C., Busch, U., and Engel, K.-H. (2009). Simultaneous detection of DNA from 10 food allergens by ligation-dependent probe amplification. *Food Addit. Contam. Part A-Chem.* **26**:409 - 418.

Eller, E., Hansen, T. K., and Bindslev-Jensen, C. (2012). Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. *Ann. Allergy Asthma Immunol.* **108**:332-336.

Faeste, C. K., Holden, L., Plassen, C., and Almli, B. (2006). Sensitive time-resolved fluoroimmunoassay for the detection of hazelnut (*Corylus avellana*) protein traces in food matrices. *J. Immunol. Methods* **314**:114-122.

FAOSTAT, (2013). Food and Agriculture Organization ProdStat Database. Available at: http://faostat.fao.org Last accession on March 2013.

FDA (2003). Administration Qualified Health Claims, letter of enforcement discretion-nuts and coronary heart disease (Docket No 02P-0505), Ingredients, Packaging & Labeling, Food and Drug Administration, Washington DC. Available at: http://www.fda.gov

Fernández-Rivas, M., and Cuevas, M. (1999). Peels of Rosaceae fruits have a higher allergenicity than pulps. *Clin. Exp. Allergy* **29**:1239-1247.

Garber, E., and Perry, J. (2010). Detection of hazelnuts and almonds using commercial ELISA test kits. *Anal. Bioanal. Chem.* **396**:1939-1945.

García, B. E., and Lizaso, M. T. (2011). Cross-reactivity syndromes in food allergy. *J. Invest. Allergol. Clin. Immunol.* **21**:162-170.

Garino, C., Zuidmeer, L., Marsh, J., Lovegrove, A., Morati, M., Versteeg, S., Schilte, P., Shewry, P., Arlorio, M., and van Ree, R. (2010). Isolation, cloning, and characterization of the 2S albumin: a new allergen from hazelnut. *Mol. Nutr. Food Res.* **54**:1257-1265.

Germini, A., Scaravelli, E., Lesignoli, F., Sforza, S., Corradini, R., and Marchelli, R. (2005). Polymerase chain reaction coupled with peptide nucleic acid high-performance liquid chromatography for the sensitive detection of traces of potentially allergenic hazelnut in foodstuffs. *Eur. Food Res. Technol.* **220**:619-624.

Gruehn, S., Suphioglu, C., O'Hehir, R. E., and Volkmann, D. (2003). Molecular cloning and characterization of hazel pollen protein (70 kD) as a luminal binding protein (BiP): a novel cross-reactive plant allergen. *Int. Arch. Allergy Immunol.* **131**:91-100.

Guo, F., Kothary, M. H., Wang, Y., Yu, X., Howard, A. J., Fu, T.-J., and Zhang, Y.-Z. (2009). Purification and crystallization of Cor a 9, a major hazelnut allergen. *Acta Crystallogr. F-Struct. Biol. Cryst. Commun.* **65**:42-46.

Gupta, R. S., Springston, E. E., Warrier, M. R., Smith, B., Kumar, R., Pongracic, J., and Holl, J. L. (2011). The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* **128**:e9-e17.

Hansen, K. S., Ballmer-Weber, B. K., Lüttkopf, D., Skov, P. S., Wüthrich, B., Bindslev-Jensen, C., Vieths, S., and Poulsen, L. K. (2003). Roasted hazelnuts - allergenic activity evaluated by double-blind, placebo-controlled food challenge. *Allergy* **58**:132-138.

Hansen, K. S., Ballmer-Weber, B. K., Sastre, J., Lidholm, J., Andersson, K., Oberhofer, H., Lluch-Bernal, M., Östling, J., Mattsson, L., Schocker, F., Vieths, S., and Poulsen, L. K. (2009). Component-resolved in vitro diagnosis of hazelnut allergy in Europe. *J. Allergy Clin. Immunol.* **123**:1134-1141.e1133.

Harrer, A., Egger, M., Gadermaier, G., Erler, A., Hauser, M., Ferreira, F., and Himly, M. (2010). Characterization of plant food allergens: an overview on physicochemical and immunological techniques. *Mol. Nutr. Food Res.* **54**:93-112.

Hartz, C., Lauer, I., San-Miguel Moncin, M., Cistero-Bahima, A., Foetisch, K., Lidholm, J., Vieths, S., and Scheurer, S. (2010). Comparison of IgE-binding capacity, cross-reactivity and biological potency of allergenic non-specific lipid transfer proteins from peach, cherry and hazelnut. *Int. Arch. Allergy Immunol.* **153**:335-346.

Hauser, M., Egger, M., Wallner, M., Wopfner, N., Schmidt, G., and Ferreira, F. (2008). Molecular properties of plant food allergens: a current classification into protein families. *The Open Immunology Journal* **1**:1-12.

Hauser, M., Roulias, A., Ferreira, F., and Egger, M. (2010). Panallergens and their impact on the allergic patient. *Allergy, Asthma & Clinical Immunology* **6**:1-14.

Heick, J., Fischer, M., Kerbach, S., Tamm, U., and Popping, B. (2011a). Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. *J. AOAC Int.* **94**:1060-1068.

Heick, J., Fischer, M., and Pöpping, B. (2011b). First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J. Chromatogr. A* **1218**:938-943.

Herman, L., Block, J. D., and Viane, R. (2003). Detection of hazelnut DNA traces in chocolate by PCR. *Int. J. Food Sci. Technol.* **38**:633-640.

Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W. R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., and Kraft, D. (1992). Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* **90**:927-936.

Holzhauser, T., Stephan, O., and Vieths, S. (2002). Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *J. Agric. Food Chem.* **50**:5808-5815.

Holzhauser, T., and Vieths, S. (1999). Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *J. Agric. Food Chem.* **47**:4209-4218.

Holzhauser, T., Wangorsch, A., and Vieths, S. (2000). Polymerase chain reaction (PCR) for detection of potentially allergenic hazelnut residues in complex food matrixes. *Eur. Food Res. Technol.* **211**:360-365.

Iniesto, E., Jiménez, A., Prieto, N., Cabanillas, B., Burbano, C., Pedrosa, M. M., Rodríguez, J., Muzquiz, M., Crespo, J. F., Cuadrado, C., and Linacero, R. (2013). Real-time PCR to detect hazelnut allergen coding sequences in processed foods. *Food Chem.* **138**:1976-1981.

Iwan, M., Vissers, Y. M., Fiedorowicz, E., Kostyra, H., Kostyra, E. b., Savelkoul, H. F. J., and Wichers, H. J. (2011). Impact of Maillard reaction on immunoreactivity and allergenicity of the hazelnut allergen Cor a 11. *J. Agric. Food Chem.* **59**:7163-7171.

Johnson, P. E., Sancho, A. I., Crevel, R. W. R., and Mills, E. N. C. (2011). Detection of allergens in foods. **In**: Food Allergens, Analysis Instrumentation and Methods, pp 13-27. Nollet, L. M. L. and van Hengel, A. J., Eds., CRC Press, Boca Raton, FL.

Kader, J. C. (1996). Lipid-Transfer Proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**:627-654.

Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Krska, R., Bremer, M., Tomkies, V., Reece, P., Danks, C., Immer, U., and Weller, M. G. (2005). Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *J. Agric. Food Chem.* **53**:3321-3327.

Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., and Waiblinger, H.-U. (2010). Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur. Food Res. Technol.* **230**:367-374.

Köppel, R., Velsen-Zimmerli, F., and Bucher, T. (2012). Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. *Eur. Food Res. Technol.* **235**:843-852.

Koppelman, S. J., Knulst, A. C., Koers, W. J., Penninks, A. H., Peppelman, H., Vlooswijk, R., Pigmans, I., van Duijn, G., and Hessing, M. (1999). Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J. Immunol. Methods* **229**:107-120.

Lauer, I., Foetisch, K., Kolarich, D., Ballmer-Weber, B. K., Conti, A., Altmann, F., Vieths, S., and Scheurer, S. (2004). Hazelnut (*Corylus avellana*) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem J.* **383**:327-334.

Leduc, V., Moneret-Vautrin, D. A., Tzen, J. T. C., Morisset, M., Guerin, L., and Kanny, G. (2006). Identification of oleosins as major allergens in sesame seed allergic patients. *Allergy* **61**:349-356.

López, E., Cuadrado, C., Burbano, C., Jiménez, M. A., Rodríguez, J., and Crespo, J. F. (2012). Effects of autoclaving and high pressure on allergenicity of hazelnut proteins. *J. Clin. Bioinformatics* **2**:12.

Lüttkopf, D., Müller, U., Skov, P. S., Ballmer-Weber, B. K., Wüthrich, B., Skamstrup Hansen, K., Poulsen, L. K., Kästner, M., Haustein, D., and Vieths, S. (2001). Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol. Immunol.* **38**:515-525.

Masthoff, L. J. N., Mattsson, L., Zuidmeer-Jongejan, L., Lidholm, J., Andersson, K., Akkerdaas, J. H., Versteeg, S. A., Garino, C., Meijer, Y., Kentie, P., Versluis, A., den Hartog Jager, C. F., Bruijnzeel-Koomen, C. A. F. M., Knulst, A. C., van Ree, R., van Hoffen, E., and Pasmans, S. G. M. A. (2013). Sensitization to Cor a 9 and Cor a 14 is highly specific for a hazelnut allergy with objective symptoms in Dutch children and adults. *J. Allergy Clin. Immunol.* **132**:393-399.

Mills, E. N. C, Jenkins, J., Marigheto, N., Belton, P. S., Gunning, A. P., and Morris, V. J. (2002). Allergens of the cupin superfamily. *Biochem. Soc. Trans.* **30**:925-929.

Mills, E. N. C., Mackie, A. R., Burney, P., Beyer, K., Frewer, L., Madsen, C., Botjes, E., Crevel, R. W. R., and Van Ree, R. (2007a). The prevalence, cost and basis of food allergy across Europe. *Allergy* **62**:717-722.

Mills, E. N. C., Madsen, C., Shewry, P. R., and Wichers, H. J. (2003). Food allergens of plant origin - their molecular and evolutionary relationships. *Trends Food Sci. Technol.* **14**:145-156.

Mills, C. E., Sancho, A. I., Moreno, J., and Kostyra, H. (2007b). The effects of food processing on allergens. **In**: Managing allergens in food, pp 117-133. Mills, C., Wichers, H. and Hoffmann-Sommergruber, K., Eds., CRC Press, Boca Raton, FL.

Monaci, L., and Visconti, A. (2009). Mass spectrometry-based proteomics methods for analysis of food allergens. *Trac-Trends Anal. Chem.* **28**:581-591.

Morano, K. A. (2007). New tricks for an old dog: the evolving world of Hsp70. *Ann. NY Acad. Sci.* **1113**:1-14.

Moreno, F. J., and Clemente, A. (2008). 2S albumin storage proteins: what makes them food allergens? *Open Biochem. J.* **2**:16-28.

Mustorp, S. L., Drømtorp, S. M., and Holck, A. L. (2011). Multiplex, quantitative, ligation-dependent probe amplification for determination of allergens in food. *J. Agric. Food Chem.* **59**: 5231-5239.

Müller, U., Lüttkopf, D., Hoffmann, A., Petersen, A., Becker, W. M., Schocker, F., Niggemann, B., Altmann, F., Kolarich, D., Haustein, D., and Vieths, S. (2000). Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *Eur. Food Res. Technol.* **212**:2-12.

Napier, J., Stobart, A. K., and Shewry, P. (1996). The structure and biogenesis of plant oil bodies: the role of the ER membrane and the oleosin class of proteins. *Plant Mol. Biol.* **31**:945-956.

NCBI, (2013). National Center for Biotechnology Information, Bethesda, MD. Available at: http://www.ncbi.nlm.nih.gov/ Last accession on March 2013.

Ortolani, C., Ballmer-Weber, B. K., Hansen, K. S., Ispano, M., Wüthrich, B., Bindslev-Jensen, C., Ansaloni, R., Vannucci, L., Pravettoni, V., Scibilia, J., Poulsen, L. K., and Pastorello, E. A. (2000). Hazelnut allergy: a double-blind, placebo-controlled food challenge multicenter study. *J. Allergy Clin. Immunol.* **105**:577-581.

Pafundo, S., Gullì, M., and Marmiroli, N. (2010). Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. *Anal. Bioanal. Chem.* **396**:1831-1839.

Palacín, A., Rivas, L. A., Gómez-Casado, C., Aguirre, J., Tordesillas, L., Bartra, J., Blanco, C., Carrillo, T., Cuesta-Herranz, J., Bonny, J. A. C., Flores, E., García-Alvarez-Eire, M. G., García-Nuñez, I., Fernández, F. J., Gamboa, P., Muñoz, R., Sánchez-Monge, R., Torres, M., Losada, S. V., Villalba, M., Vega, F., Parro, V., Blanca, M., Salcedo, G., and Díaz-Perales, A. (2012). The involvement of thaumatin-like proteins in plant food cross-reactivity: a multicenter study using a specific protein microarray. *PLoS ONE* **7**(9):e44088.

Pastorello, E. A., Vieths, S., Pravettoni, V., Farioli, L., Trambaioli, C., Fortunato, D., Lüttkopf, D., Calamari, M., Ansaloni, R., Scibilia, J., Ballmer-Weber, B. K., Poulsen, L. K., Wütrich, B., Hansen, K. S., Robino, A. M., Ortolani, C., and Conti, A. (2002). Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J. Allergy Clin. Immunol.* **109**:563-570.

Pele, M., Brohee, M., Anklam, E., and Van Hengel, A. J. (2007). Peanut and hazelnut traces in cookies and chocolates: relationship between analytical results and declaration of food allergens on product labels. *Food Addit. Contam.* **24**:1334-1344.

Picariello, G., Mamone, G., Addeo, F., and Ferranti, P. (2011). The frontiers of mass spectrometry-based techniques in food allergenomics. *J. Chromatogr. A* **1218**:7386-7398.

Piknová, L., Pangallo, D., and Kuchta, T. (2008). A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. *Eur. Food Res. Technol.* **226**:1155-1158.

Pilolli, R., Monaci, L., and Visconti, A. (2013). Advances in biosensor development based on integrating nanotechnology and applied to food-allergen management. *Trends Anal. Chem.* **47**:12-26.

Platteau, C., Cucu, T., Taverniers, I., Devreese, B., De Loose, M., and De Meulenaer, B. (2012). Effect of oxidation in the presence or absence of lipids on hazelnut and soybean protein detectability by commercial ELISA. *Food Agric. Immunol.* **24**:179-192.

Platteau, C., De Loose, M., De Meulenaer, B., and Taverniers, I. (2011a). Quantitative detection of hazelnut (*Corylus avellana*) in cookies: ELISA versus real-time PCR. *J. Agric. Food Chem.* **59**:11395-11402.

Platteau, C., De Loose, M., De Meulenaer, B., and Taverniers, I. (2011b). Detection of allergenic ingredients using real-time PCR: a case study on hazelnut (*Corylus avellana*) and soy (*Glycine max*). *J. Agric. Food Chem.* **59**:10803-10814.

Poms, R. E., Klein, C. L., and Anklam, E. (2004). Methods for allergen analysis in food: a review. *Food Addit. Contam.* **21**:1-31.

Pons, L., Chery, C., Romano, A., Namour, F., Artesani, M. C., and Guéant, J. L. (2002). The 18 kDa peanut oleosin is a candidate allergen for IgE-mediated reactions to peanuts. *Allergy* **57**:88-93.

Rebe Raz, S., Liu, H., Norde, W., and Bremer, M. G. E. G. (2010). Food allergens profiling with an imaging surface plasmon resonance-based biosensor. *Anal. Chem.* **82**:8485-8491.

Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004, *Off. J. Eur. Union* **L304**:18-63.

Rejeb, S. B., Abbott, M., Davies, D., Cléroux, C., and Delahaut, P. (2005). Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Addit. Contam.* **22**:709-715.

Rejeb, S. B., Abbott, M., Davies, D., Querry, J., Cléroux, C., Streng, C., Delahaut, P., and Yeung, J. M. (2003). Immunochemical-based method for detection of hazelnut proteins in processed foods. *J. AOAC Int.* **86**:557-563.

Rigby, N. M., Marsh, J., Sancho, A. I., Wellner, K., Akkerdaas, J., van Ree, R., Knulst, A., Fernández-Rivas, M., Brettlova, V., Schilte, P. P., Summer, C., Pumphrey, R., Shewry, P. R., and Mills, E. N. C. (2008). The purification and characterisation of allergenic hazelnut seed proteins. *Mol. Nutr. Food Res.* **52**(S2):S251-S261.

Robotham, J. M., Hoffman, G. G., Teuber, S. S., Beyer, K., Sampson, H. A., Sathe, S. K., and Roux, K. H. (2009). Linear IgE-epitope mapping and comparative structural homology modeling of hazelnut and English walnut 11S globulins. *Mol. Immunol.* **46**:2975-2984.

Röder, M., Vieths, S., and Holzhauser, T. (2009). Commercial lateral flow devices for rapid detection of peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) cross-contamination in the industrial production of cookies. *Anal. Bioanal. Chem.* **395**:103-109.

Rossi, S., Scaravelli, E., Germini, A., Corradini, R., Fogher, C., and Marchelli, R. (2006). A PNA-array platform for the detection of hidden allergens in foodstuffs. *Eur. Food Res. Technol.* **223**:1-6.

Roux, K. H., Teuber, S. S., and Sathe, S. K. (2003). Tree nut allergens. *Int. Arch. Allergy Immunol.* **131**:234-244.

Salcedo, G., Sanchez-Monge, R., Barber, D., and Diaz-Perales, A. (2007). Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochim. Biophys. Acta* **1771**:781 - 791.

Sancho, A. I., Rigby, N. M., Zuidmeer, L., Asero, R., Mistrello, G., Amato, S., González-Mancebo, E., Fernández-Rivas, M., van Ree, R., and Mills, E. N. C. (2005). The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* **60**:1262-1268.

Scheibe, B., Weiss, W., Ruëff, F., Przybilla, B., and Görg, A. (2001). Detection of trace amounts of hidden allergens: hazelnut and almond proteins in chocolate. *J. Chromatogr. B - Biomed. Sci. Appl.* **756**:229-237.

Schocker, F., Lüttkopf, D., Müller, U., Thomas, P., Vieths, S., and Becker, W. M. (2000). IgE binding to unique hazelnut allergens: identification of non-pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions. *Eur. J. Nutr.* **39**:172-180.

Schocker, F., Lüttkopf, D., Scheurer, S., Petersen, A., Cisteró-Bahima, A., Enrique, E., San Miguel-Moncín, M., Akkerdaas, J., van Ree, R., Vieths, S., and Becker, W.-M. (2004). Recombinant lipid transfer protein Cor a 8 from hazelnut: a new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J. Allergy Clin. Immunol.* **113**:141-147.

Schöringhumer, K., Redl, G., and Cichna-Markl, M. (2009). Development and Validation of a Duplex Real-time PCR method to simultaneously detect potentially allergenic sesame and hazelnut in food. *J. Agric. Food Chem.* **57**:2126-2134.

Schubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Führer, M., Molinelli, A., and Baumgartner, S. (2009). Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Anal. Bioanal. Chem.* **395**:69-81.

Schulten, V., Lauer, I., Scheurer, S., Thalhammer, T., and Bohle, B. (2011a). A food matrix reduces digestion and absorption of food allergens in vivo. *Mol. Nutr. Food Res.* **55**:1484-1491.

Schulten, V., Nagl, B., Scala, E., Bernardi, M. L., Mari, A., Ciardiello, M. A., Lauer, I., Scheurer, S., Briza, P., Jürets, A., Ferreira, F., Jahn-Schmid, B., Fischer, G. F., and Bohle, B. (2011b). Pru p 3, the nonspecific lipid transfer protein from peach, dominates the immune response to its homolog in hazelnut. *Allergy* **66**:1005-1013.

Shewry, P. R., Napier, J. A., and Tatham, A. S. (1995). Seed storage proteins: structures and biosynthesis. *Plant Cell* **7**:945-956.

Sicherer, S. H., Muñoz-Furlong, A., Godbold, J. H., and Sampson, H. A. (2010). US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol.* **125**:1322-1326.

Sicherer, S. H., Muñoz-Furlong, A., and Sampson, H. A. (2003). Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **112**:1203-1207.

Sicherer, S. H., and Sampson, H. A. (2006). Food allergy. *J. Allergy Clin. Immunol.* **117**(2, Suppl. 2):S470-S475.

Sicherer, S. H., and Sampson, H. A. (2009). Food allergy: recent advances in pathophysiology and treatment. *Annu. Rev. Med.* **60**:261-277.

Sicherer, S. H., and Sampson, H. A. (2010). Food allergy. *J. Allergy Clin. Immunol.* **125**(2, Suppl. 2):S116-S125.

Soller, L., Ben-Shoshan, M., Harrington, D. W., Fragapane, J., Joseph, L., St. Pierre, Y., Godefroy, S. B., La Vieille, S., Elliott, S. J., and Clarke, A. E. (2012). Overall prevalence of self-reported food allergy in Canada. *J. Allergy Clin. Immunol.* **130**:986-988.

Stanley, J. S., and Bannon, G. A. (1999). Biochemistry of food allergens. *Clin. Rev. Allergy Immunol.* **17**:279-291.

Stephan, O., Möller, N., Lehmann, S., Holzhauser, T., and Vieths, S. (2002). Development and validation of two dipstick type immunoassays for determination of trace amounts of peanut and hazelnut in processed foods. *Eur. Food Res. Technol.* **215**:431-436.

Taylor, S. L., Crevel, R. W. R., Sheffield, D., Kabourek, J., and Baumert, J. (2009). Threshold dose for peanut: Risk characterization based upon published results from challenges of peanut-allergic individuals. *Food Chem. Toxicol.* **47**:1198-1204.

Tortajada-Genaro, L. A., Santiago-Felipe, S., Morais, S., Gabaldón, J. A., Puchades, R., and Maquieira, Á. (2012). Multiplex DNA detection of food allergens on a digital versatile disk. *J. Agric. Food Chem.* **60**:36-43.

Trashin, S. A., Cucu, T., Devreese, B., Adriaens, A., and De Meulenaer, B. (2011). Development of a highly sensitive and robust Cor a 9 specific enzyme-linked immunosorbent assay for the detection of hazelnut traces. *Anal. Chim. Acta* **708**:116-122.

Tzen, J. T., Lie, G. C., and Huang, A. H. (1992). Characterization of the charged components and their topology on the surface of plant seed oil bodies. *J. Biol. Chem.* **267**:15626-15634.

UniProt, (2013). Protein knowledgebase, UniProt Consortium. Available at: http://www.uniprot.org/ Last accession March 2013.

USDA, (2013). United States Department of Agriculture - Germplasm Resources Information Network, Beltsville, MD. Available at: http://www.ars-grin.gov/ Last accession on March 2013.

Valenta, R., Duchene, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edelmann, H., and Kraft, D. (1992). Profilins constitute a novel family of functional plant pan-allergens. *J. Exp. Med.* **175**:377-385.

Verweij, M. M., Hagendorens, M. M., De Knop, K. J., Bridts, C. H., De Clerck, L. S., Stevens, W. J., and Ebo, D. G. (2011). Young infants with atopic dermatitis can display sensitization to Cor a 9, an 11S legumin-like seed-storage protein from hazelnut (*Corylus avellana*). *Pediatr. Allergy Immunol.* **22**:196-201.

Vieths, S., Scheurer, S., and Ballmer-Weber, B. (2002). Current understanding of cross-reactivity of food allergens and pollen. *Ann. NY Acad. Sci.* **964**:47-68.

Wang, W., Han, J., Wu, Y., Yuan, F., Chen, Y., and Ge, Y. (2011). Simultaneous detection of eight food allergens using optical thin-film biosensor chips. *J. Agric. Food Chem.* **59**:6889-6894.

Yman, I. M., Eriksson, A., Johansson, M. A., and Hellenas, K. E. (2006). Food allergen detection with biosensor immunoassays. *J. AOAC Int.* **89**:856-861.

Zuidmeer, L., Goldhahn, K., Rona, R. J., Gislason, D., Madsen, C., Summers, C., Sodergren, E., Dahlstrom, J., Lindner, T., Sigurdardottir, S. T., McBride, D., and Keil, T. (2008). The prevalence of plant food allergies: a systematic review. *J. Allergy Clin. Immunol.* **121**:1210-1218.e1214.

Zuidmeer, L., and van Ree, R. (2007). Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. *Curr. Opin. Allergy Clin. Immunol.* **7**:269-273.

EXPERIMENTAL PART

Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut

Journal of Agricultural and Food Chemistry, **2012**, 60, 8103-8110

Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates

Food Chemistry, (submitted)

Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR

Analytical and Bioanalytical Chemistry (submitted)

ACS Publications © 2012 American Chemical Society

J. Agric. Food Chem. 2012, 60, 8103−8110

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut

Joana Costa,[†] Isabel Mafra,[*,†] Tomas Kuchta,[‡] Maria Beatriz P.P. Oliveira[†]

[†]REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal. [‡]Department of Microbiology and Molecular Biology, VUP Food Research Institute, Bratislava, Slovakia.

*Corresponding author: Tel: +351 220428640. Fax: +351 226093390. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

Hazelnut is one of the most commonly consumed tree nuts, being largely used by the food industry in a wide variety of processed foods. However, it is a source of allergens capable of inducing mild to severe allergic reactions in sensitized individuals. Hence, the development of highly sensitive methodologies for hazelnut traceability is essential. In this work, we developed a novel technique for hazelnut detection based on single-tube nested real-time PCR system. The system presents high specificity and sensitivity, enabling a relative limit of detection of 50 mg/kg of hazelnut in wheat material and an absolute limit of detection of 0.5 pg of hazelnut DNA (1 DNA copy). Its application to processed food samples was successfully achieved, detecting trace amounts of hazelnut in chocolate down to 60 mg/kg. These results highlight the adequacy of the technique for the specific detection and semi-quantitation of hazelnut as potential hidden allergens in foods.

Keywords: hazelnut detection, single-tube nested real-time PCR, tree nuts, food allergens

INTRODUCTION

Food-induced allergic reactions are considered an emerging problem of public health with special impact in industrialized countries. They are defined as adverse, immune-mediated (IgE mediated) responses to the presence of offending food ingredients, namely proteins and glycoproteins, in sensitized individuals.[1] In recent years, the prevalence of food-induced allergies seems to be rising and it is estimated to affect almost 3-4% of adult population and 6% of young children.[1] A large range of foods are known to be responsible for triggering allergies, but the majority of the allergic reactions can be attributed to a specific number of foods, in which are included the tree nuts.[2]

Hazelnut (*Corylus avellana* L.) is one of the most commonly consumed tree nuts. It is well appreciated either raw or roasted, and has a wide application, namely in bakery and chocolate formulations. In Europe, hazelnut allergy is very frequent and is often related to birch pollinosis.[3] Clinical manifestations caused by hazelnut ingestion in allergic patients can vary from oral allergy syndrome and/or gastrointestinal symptoms, to fatal reactions (anaphylactic shock), mainly in children and adolescents.[4,5] Recently, in a study reported by EuroPrevall, involving a large multicountry sample, namely USA, Australia and eleven countries from Europe, hazelnut allergy was estimated with an overall prevalence of 7.2%.[6] Moreover, when birch allergic subjects were excluded from this test population, hazelnut remained the second most frequent food component inducing allergy with an overall incidence of 3.1%.[6] The allergen doses capable of inducing perceptible symptoms in sensitized patients are difficult to assess, however threshold doses producing subjective reactions can range from 1 mg up to 100 mg of hazelnut protein that is equivalent to 6.4-640 mg of hazelnut meal.[4] Because these threshold levels are comparable to those potentially hidden in dietary food products, restriction labeling and more accurate strategies to prevent and detect contamination of foods with hazelnut traces should be implemented.

Currently, the majority of analytical tools for the detection and quantitation of allergens in foods target either proteins or DNA.[7] Regarding hazelnut, several analytical approaches have been used. The enzyme-linked immunosorbent assays (ELISA) are the most largely applied techniques due to their capacity to directly monitor the actual allergens or the marker proteins of the species.[8-11] The direct detection of the allergenic proteins in foods is regarded as one of the most commonly applied, so the immunochemical assays such as ELISA and lateral flow devices continues to be widely used. In general, this type of assays allows reaching relatively low limits of detection (LOD) (10 ppm of hazelnut protein in cookies[8] and 1 ppm of hazelnut extract in doughs[9]), being considered of rapid performance. However, the use of immunoassays faces numerous problems essentially related to cross-reactivity with non-target proteins and their low resistance to food processing.[12] More recently, the mass spectrometry-based methods have also been used as protein identifying techniques.[13-15] Liquid chromatography coupled with the mass spectrometry approach has been recently applied to the detection and quantitation of proteins in foods with the clear advantage of directly monitoring the target peptides without depending on the antibody protein interaction such as in case of immunoassays. Until now this technique has been applied to detect hazelnut proteins in foods with high level of sensitivity (5 mg/kg of target peptide with 13 amino acid length in bread,[14] 30-35 mg/kg of Cor a 9 peptide with 5 amino acid length in five nut mixture[15]). The high cost of the equipment and the complexity of data to analyze are drawbacks associated to mass-spectrometry methods. Considering the higher stability of DNA to food processing and a lower probability of cross-reactivity, the techniques based on polymerase chain reaction (PCR) have also attained an essential role for the detection of allergens in foods. Species-specific PCR and real-time PCR approaches have been advanced as alternative tools for the indirect detection of hazelnut allergens in food products.[16-21] Until now the reported methods for the detection and quantitation of hazelnut in foods by real-time PCR allow attaining a relative LOD of 100 mg/kg of hazelnut in flour[18] and in walnut[21], and an absolute LOD of 5 to 13 pg of hazelnut.[16,18,19] Concerning the low levels of hazelnut needed to induce an allergic reaction in sensitized consumers, the development of new and more sensitive methodologies is crucial.

In this work, we applied a novel approach for hazelnut detection based on single-tube nested real-time PCR. The developed method was based on the same principle reported by Bergerová et al.[22] for peanut detection, assembling the advantages of two PCR techniques, namely nested PCR and real-time PCR. The nested PCR technique has been widely used to increase sensitivity, reduce amplification of non-specific DNA target and to enable isolation or identification of specific product.[23] The common procedure for its application is based on two sequential and distinct PCR amplifications, where in the first reaction the outer primers enable the production of fragments that will serve as the DNA template for the second reaction. However, the performance of conventional nested PCR to enhance the specificity and the production of target fragments also bears two major disadvantages: increased possibility of cross-contamination and higher number of manipulations than in one-round PCR. To overcome these drawbacks, the development of closed tube reactions containing both the outer (first PCR) and inner (second PCR) primers has been attempted.[23] One means of performing closed tube reactions consists of initial PCR cycles at high annealing temperatures followed by later cycles at low hybridization temperatures, combined in the same reaction. This approach has been applied for both end-point[23] and real-time PCR.[22-24] The technique consists of combining the use of two pairs of nested primers with different annealing temperatures coupled with real-time PCR technology in a single reaction. Thus, the two pairs of primers allow the production of two specific DNA fragments because they anneal at distinct temperatures. The second fragment produced with nested (inner) primers is directly monitored by the use of real-time PCR and a specific probe. This system eliminates the original problems of cross-contamination related with routine use of the nested reaction. It also introduces higher specificity to the method conferred by two pairs of primers and a real-time fluorogenic probe in a single reaction tube, which remains closed throughout the entire analysis. The application of nested real-time PCR system to detect trace amounts of hazelnut in foods was aimed at enhancing the sensitivity of the technique by at least one order of magnitude, regarding the available methods described in the literature.

MATERIALS AND METHODS

Plant foods and sample preparation. A total of 18 cultivars of hazelnut (Morell, Negret, Grossal, Buttler, Ennis, Pauetet, Fertile de Coutard, Segorbe, Sta María del Gésu, Tonda di Giffoni, Culplà, Merveille de Bollwiller, Camponica, Lunga di Spagna, Cosford, Gunslebert, Round du Piémont and Lansing) were collected in an experimental orchard at Vila Real, in the north region of Portugal. Hazelnut, other tree nuts that included walnut, macadamia nut, almond, Brazil nut, chestnut, cashew, pistachio and peanut, and different plant foods (soybean, lupine, fava bean, maize, oat, barley, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, apricot, plum, cherry, strawberry and raspberry) were obtained at local markets. Processed food samples were also obtained at local markets comprising 18 different chocolates containing hazelnuts and/or almonds as well as plain chocolates, and a sample of breakfast cereals with nuts.

Due to the lack of reference or testing materials for hazelnut detection, binary model mixtures containing 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5% and 10% of commercial hazelnut in wheat material (triturated pasta) were prepared. The first sample spiked with 10% of hazelnut was prepared by adding 20 g of hazelnut to 180 g of pasta, performing a complete homogenization of the mixture. All the other model mixtures were serially diluted by successive additions of wheat material until 10 mg/kg (0.001%) in the equivalent proportion.

All plant and processed food samples, as well as reference mixtures were ground and homogenized separately, into a fine powder of approximately 0.3 mm of diameter in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) using different containers and material, previously treated with a DNA decontamination solution. The fruits, namely tomato, peach, apricot, plum, cherry, strawberry and raspberry, were lyophilized before grinding.

After preparation, all samples and reference mixtures were immediately stored at -20 ºC until further DNA extraction.

DNA extraction. DNA was extracted from all samples by chaotropic solid-phase extraction using the commercial Nucleospin Food Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions with minor alterations. Briefly, to 200 mg of sample, 700 µL of CF lysis solution pre-heated at 65 ºC and 10 µL of proteinase K (20 mg/mL) were added. After an incubation period of 1 h at 65 ºC with continuous stirring, 4 µL of RNase A (10 mg/mL) were added to each mixture, being submitted to a new incubation for 10 min at 37 ºC with gentle stirring. The samples were centrifuged for 10 min (18,500*g* at 4 ºC) and 550 µL of supernatant were transferred to a new sterile reaction tube. The supernatant was then submitted to a new centrifugation step for 10 min (18,500*g* at 4 ºC). Approximately 450 µL of supernatant were removed to a new reaction tube, where C4 precipitation solution and ethanol 100% were added in equal volumes to the supernatant. Each mixture was homogenized by gentle inversion and all the volume eluted through one spin column by centrifugation (1 min, 13,000*g* at room temperature). The spin column was washed three times with 400 µL of CQW solution, 700 µL and 200 µL of C5 solution, followed by 1 min of centrifugation after the two first washes and a 2 min final centrifugation (13,000*g* at room temperature). DNA was eluted from the column by adding 100 µL of CE solution at 70 ºC, followed by 5 min incubation at room temperature and centrifugation (1 min, 13,000*g*). All the DNA isolates were kept at -20 ºC until further analysis. The extractions were performed at least in duplicate for each sample.

Yield and purity of extracts were assessed by 1% agarose gel electrophoresis and by UV spectrophotometry using a spectrophotometer UV1800 (Shimadzu, Kyoto, Japan).

Target gene selection and oligonucleotide primers. The sequence corresponding to *Corylus avellana* low molecular weight heat-shock protein (HSP1) mRNA, complete cds was retrieved from Genbank database (accession no. AF021807.1). Two sets of specific primers were designed using the software Primer-BLAST designing tool[25] (Table 1). The software parameters were set to design the first pair of primers (Hsp1F/Hsp1R) with an optimal annealing temperature (Ta) around 66 ºC. The second pair of primers was designed to anneal at a lower Ta (54 ºC), considering a difference of at least 10 ºC between the two sets of primers. The hydrolysis probe was the same as in the original TaqMan system (Table 1).[21] All oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Sequencing. For sequencing the region to be amplified, a third set of primers (Hsp3F/Hsp3R) was specifically designed to produce larger fragments (323 bp), encompassing the target region of 126 bp defined by the pair of primers Hsp1F/Hsp1R, using the end-point PCR conditions described below. The amplified fragments of ten hazelnut samples, comprising nine different cultivars and the commercial hazelnut sample used to prepare the model mixtures were sequenced. All PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove interfering components and sequenced in a specialized research facility (STABVIDA, Lisbon, Portugal). Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two high quality complementary sequences.

Table 1. Primers and Hydrolysis Probe Targeting *Corylus avellana* Low Molecular Weight Heat-Shock Protein (HSP1) mRNA Sequence.

Oligonucleotides	Sequence (5'-3')	Amplicon (bp)	References
Outer primers			
Hsp1F	AGC GTC GAG AGT GGC AAG TTC		This work[a]
Hsp1R	CCT GCT CGC CTC CGC TTT C	126	
Nocc1P	FAM-CCT GAC GAT GCG ATG CTC GAC CAG-BHQ2		Piknová et al.[21]
Inner primers			
Hsp2F	AGT TCG TGA GCA GGT TCA	97	This work[a]
Hsp2R	GCT TTC GGA ATA GTC ACA		
Sequencing primers			
Hsp3F	CAC GTG CTG AAG GCT TCT CTT C	323	This work[a]
Hsp3R	AGG AGC TCA CGA TAA CCT TCA ACA		

[a] Genbank accession no. AF021807.1

End-point PCR. PCR amplification was carried out in 25 µL of total reaction volume containing 2 µL of DNA extract of hazelnut (100 ng), 670 mM of Tris-HCl (pH 8.8), 160 mM of $(NH4)_2SO_4$, 0.1% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3 mM of $MgCl_2$ and 200 nM of each primer Hsp3F/Hsp3R (Table 1). The reactions were performed in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA) using the following program: initial denaturation at 95 ºC for 5 min; 40 cycles at 95 ºC for 30 s, 65 ºC for 45 s and 72 ºC for 1 min; and a final extension at 72 ºC for 5 min.

Real-time PCR assays. Real-time PCR assays were performed in 20 µL of total reaction volume. Each reaction tube comprised 2 µL of DNA (100 ng), 1x of SsoFast Probes Supermix (Bio-Rad, Hercules, CA), 200 nM of each primer Hsp1F/Hsp1R and 100 nM of hydrolysis probe Nocc1P (Table 1). For nested real-time PCR amplification, the mix included additionally 200 nM of the primers Hsp2F/Hsp2R, specifically designed for this assay. All real-time PCR assays were made on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA). Real-time PCR amplifications based on the conventional technique were performed according to the following temperature protocol: 95 ºC for 5 min, 50 cycles at 95 ºC for 15 s and 66 ºC for 45 s, with the collection of fluorescence signal at the end of each cycle. Nested real-time PCR assays were done in two different phases. Phase 1: 95 ºC for 5 min, 10 or 14 cycles at 95 ºC for 15 s and 66 ºC for 45 s. During phase 2, the collection of the fluorescence signal was made at the end of each cycle: 36 or 40 cycles at 95 ºC for 15 s, 54 ºC for 20 s and 72 ºC for 30 s. Data were collected and analyzed using the software Bio-Rad CFX Manager 2.1 (Bio-Rad, Hercules, CA). Cycle threshold (Ct) values were calculated using the software at automatic threshold setting. Real-time PCR and nested real-time PCR trials were repeated two and three times using four replicates, respectively.

Application of the single-tube nested real-time PCR system to commercial foods. The amplifications by real-time PCR were carried out in 20 µL of total reaction volume containing 2 µL of DNA extract of hazelnut reference mixtures (100 ng) or commercial foods (20 ng) and the reaction components and temperature program described above for nested real-time PCR. The assays were performed triplicate for standard reference mixtures and commercial samples.

RESULTS AND DISCUSSION

In order to answer to the increasing demand for more sensitive and accurate methods for allergen detection, we propose a new real-time PCR system based on the single-tube nested real-time PCR for the detection of hazelnut in food products. In this study, *hsp1* gene encoding the heat shock protein hsp1 was the chosen target for the detection and semi-quantitation of hazelnut. On the basis of a reported work applied to the detection of microorganism *Crystosporidium parvum*[24] and, more recently, to the detection of peanut allergen Ara h 3,[22] a new single-tube nested real-time PCR system has been developed aiming to trace minute amounts of hazelnut.

For this novel technique, two sets of primers were designed. The first set producing PCR fragments of 126 bp were used as “outer” primers to define the chosen target sequence (Figure 1, Table 1).

In this system, primers Hsp1F/Hsp1R and probe Nocc1P were selected to anneal at relatively high hybridization temperatures (66 ºC) that conferred great selectivity to the reaction. The second pair of primers (Hsp2F/Hsp2R), generating PCR fragments of 97 bp, were set to act as “inner” primers functional at lower hybridization temperatures (54 ºC). The successful empirical rule for single-tube nested real-time PCR system for Ara h 3 detection[22] based on T_a (inner primers) < T_a (outer primers) < T_a (probe) was also followed to propose a method for hazelnut detection. The temperature program was defined using two phases. Phase 1 was used to amplify PCR fragments of 126 bp without collection of fluorescence at the end of each cycle. This phase 1 aimed at producing fragments that would serve as template for the second phase of the protocol. Therefore, phase 1 was optimized using different number of cycles ranging from 10 to 20 (data not shown), being the best results achieved using 10 and 14 amplification cycles in this phase. The entire protocol of reactions was always set to a total of 50 cycles, so in phase 2, the number of cycles ranged from 36 to 40.

1 GTCAATTTGGCAAAGATGTCGATCGTCCCAAACAATGAGCGAGAGCGCAGTGTCTCCAAT
61 CCCTCCTCCAGAGACCTGTGGGACGTCTTCCGGAGCTTCAGAGAGAACCACCTTCAGGAC
121 CCATTCAGCGATTTACCTTTCGCTTCTACACTCTCCACGCTCTTCCCTCACTCCCCGTTC
181 GGGAGCTCGGTGAACACCAGGCTCGACTGGAGGGAGACCCCGAGAGCCCACGTGCTGAAG
241 GCTTCTCTTCCGGGGTTCGTGGACGAGGACGTGTTGGTGGAGCTCCAAGACGACCGAGTG
301 CTCCAGGTG**AGCGTCGAGAGTGGCAAGTTC**GTGAGCAGGTTCAAGGTC**CCTGACGATGCG**
361 **ATGCTCGACCAG**TTGAAGGCCTCGATGCACAATGGGGTTCTCACTGTGACTATTCC**GAAA**
421 **GCGGAGGCGAGCAGG**CCAACCGTTCGGACCATCGAGATCTCTGGCTAAATATGTGATCTT
481 CCTTTGCCCTGTCTGTCACTATGTTTATATGTTGTGTGTGTGTGGGTTGTTGAAGGTTAT
541 CGTGAGCTCCTCTGAGTTTGTTATGAAGGTGTTTGATAAAATGCGTCTAAAGTTTGTGAT
601 GTTTGTGTCGGTTCTTGTGATGTTGACCCTTTGCTTAGTGTGTCTTAAATGGTTGTTGGG
661 TTGAGAAGAAAATACACTATGTTGTTAACAACAAAAAAAAAAAAAAAAAAAAAAAAAAAA
721 AAA

Figure 1. Region of the *hsp1* gene encoding the heat shock protein (hsp1) of hazelnut (Genbank accession no. AF021807.1). "Outer" primers (Hsp1F/Hsp1R) are exhibited in bold, "inner" primers (Hsp2F/Hsp2R) are underlined and probe (Nocc1P) is displayed in bold and double-underlined. Primers (Hsp3F/Hsp3R) used for sequencing are shaded in grey.

Sequencing of PCR products for identity conformation. The fragments of 126 bp produced in PCR systems were rather short for accurate direct sequencing, since the platform used often does not allow perfect resolution for the reading of the first 50 bp at the 5'-end of the sequence. To overcome this drawback, some strategies can be used, such as cloning amplicons into a vector. More recently, on the basis of the sequencing of highly degraded DNA fragments from fossil specimens,[26] the use of primers with a nonspecific tail of 60 nucleotide bases in the 5'-end was attempted to improve the quality of the sequencing of small hazelnut amplicons.[18] To avoid cloning PCR products into a vector or developing sequencing primers with long tails that are much more expensive, a third set of primers (Hsp3F/Hsp3R) was specifically designed to produce longer fragments encompassing the target region (Figure 1). This approach was considered easy, simple and reliable since, by the production of 323 bp amplicons and direct sequencing, it was possible to obtain the complete and accurate information of the target 126 bp amplicon without any errors.

The results of sequencing the ten hazelnut samples (9 different cultivars and a commercial sample) presented high resolution electropherograms, thus showing the adequacy of the chosen strategy. The method allowed the sequencing of the fragments in all the extension of the target area, with no differences encountered among all the tested hazelnut samples (data not shown). The sequenced fragments were also aligned with the hsp1 gene sequence from GenBank, exhibiting 100% homology.

Specificity. Prior to the specific amplification of hazelnut, the DNA extracts were evaluated for their amplifiability with universal eukaryotic primers 18SEUDIR/18SEUINV.[27] All samples tested positively with the universal primers, confirming the absence of false negative results that might occur due to possibility of PCR inhibition or ineffective DNA extraction.

The specificity of the designed primers for the target sequence (hsp1 gene) was extensively evaluated using eighteen hazelnut cultivars and a commercial hazelnut sample used to prepare the standards, other tree nuts and several plant species by PCR. The results for the specificity and cross-reactivity of the selected primers are presented as supporting information. Only the 19 hazelnut samples presented positive amplification with the designed primers. No positive amplification was observed for any other tree nuts or plant species. In contrast, cross-reactivity between hazelnut and other plant species such as strawberry, raspberry and pistachio has been described by other authors for commercially available PCR assays.[18] In this study, samples of pistachio nuts, raspberry and strawberry fruits were also included, however no cross-reactivity was observed for those samples with the proposed primers for hazelnut identification. These data evidenced the adequacy of the chosen target sequence for the development of species-specific PCR methods regarding the detection of hazelnut in foods.

Analytical method evaluation. _Real-time PCR system._ To optimize the conditions for real-time PCR amplification, DNA extracts from binary reference mixtures containing known amounts of hazelnut in wheat material were used in both systems (real-time PCR and single-tube nested real-time PCR). The prerequisites for the evaluation and comparison of the real-time PCR systems were based on the available document of the definition of minimum performance requirements for analytical methods of genetically modified organisms (GMO) testing,[28] since no requirements are yet defined for allergen testing.

The real-time PCR assays were performed using reference mixtures ranging from 10% to 0.001% of hazelnut in wheat material. The application of this system to model mixtures allowed establishing a relative limit of detection (LOD) of 0.01% (100 mg/kg) of hazelnut in wheat material (Figure 2A, Table 2). This sensitivity value was determined accounting with the total number of positive replicates in all the performed real-time PCR assays, since analytical methods should detect the presence of the target analyte at least 95% of the time at the LOD, thus ensuring less than 5% of false negative results.[28,29]

Table 2. Results of Detection of Spiked Hazelnut in Reference Model Mixtures by Two Different PCR Systems (Real-Time PCR and Nested Real-Time PCR).

	Real-time PCR system	Nested real-time PCR system
Spiked level (mg/kg)	Ct ± SD [a]	Ct ± SD [a]
10	nd [b]	nd
50	nd	27.29 ± 0.59 (12)
100	37.16 ± 0.78 (8)	27.02 ± 1.28 (12)
500	35.32 ± 1.06 (8)	24.90 ± 0.72 (12)
1,000	34.84 ± 0.76 (8)	23.50 ± 0.60 (12)
5,000	32.03 ± 0.28 (8)	21.56 ± 0.31 (12)
10,000	31.07 ± 0.22 (8)	20.28 ± 0.23 (12)
50,000	28.72 ± 0.19 (8)	18.28 ± 0.10 (12)
100,000	27.40 ± 0.15 (8)	17.14 ± 0.05 (12)
Correlation coefficient (R^2)	0.9926	0.9956
Slope	-3.3022	-3.1650
PCR efficiency (%)	100.8	107.0

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (n=8) and (n=12). [b] nd, not detected.

According to the definition of minimum performance requirements, the correlation coefficient (R^2) of standard curves should be above 0.98 and PCR efficiency between 90% and 110%, which implicates slopes ranging from -3.6 and -3.1, respectively.[28] All the assays performed with real-time PCR system presented high values of correlation and efficiency. The parameters of the reactions exhibited average values for R^2 of 0.9926, slope of -3.302 and PCR efficiency of 100.8% (Figure 2C, Table 2), being in good accordance with the acceptance criteria for method performance.[28,29] The mean value of Ct established for the lowest amplified standard (0.01%) corresponded to 37.17 cycles (Table 2).

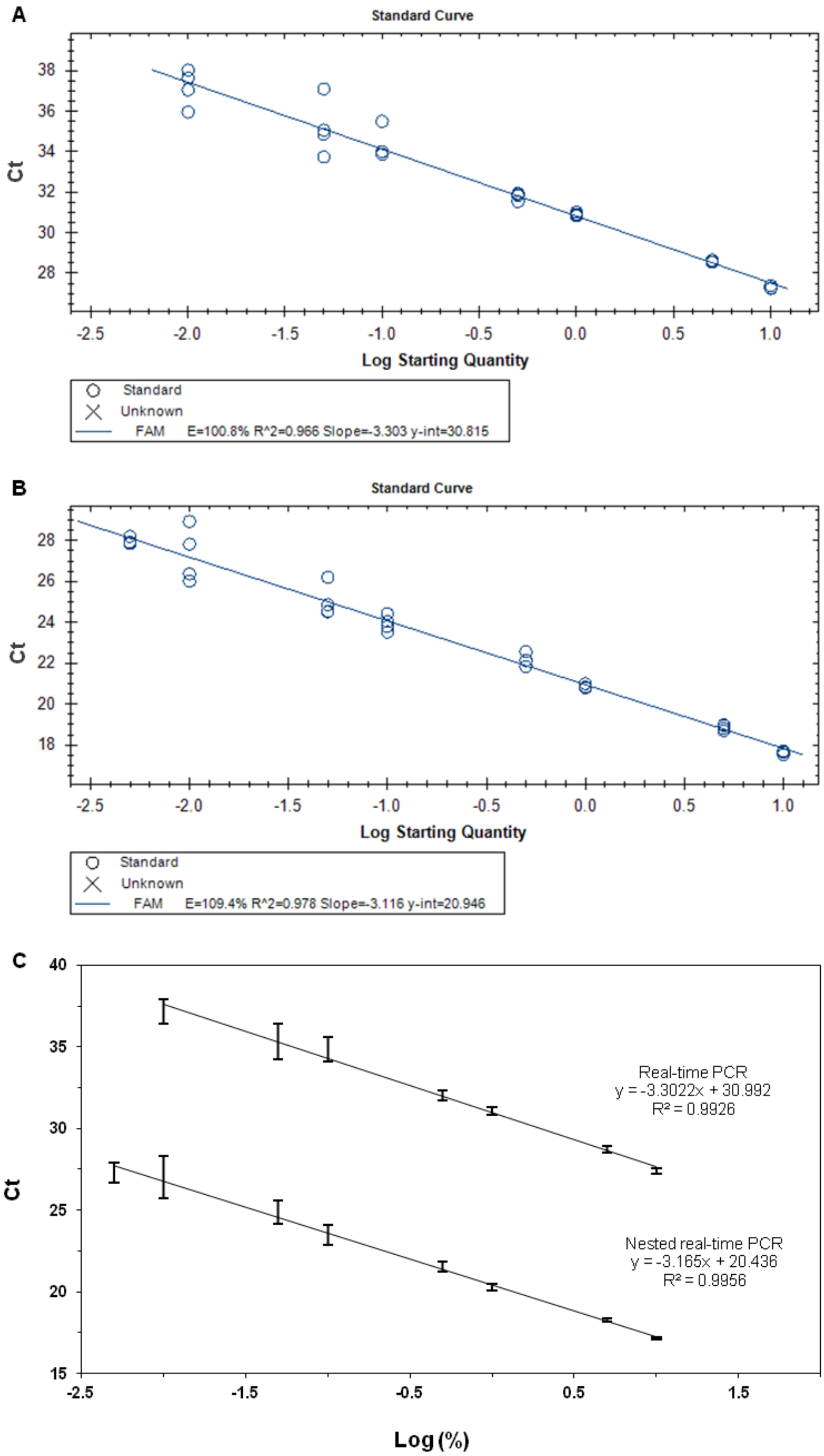


Figure 2. Calibration curves for real-time PCR (A) and nested real-time PCR (B) systems of reference binary mixtures containing 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005% and 0.001% of hazelnut in wheat material. Average values and corresponding standard deviations of *n*=8 and *n*=12 replicates for real-time PCR and nested real-time PCR, respectively (C).

To establish the dynamic range and the absolute limit of detection, 10-fold serially dilutions of hazelnut DNA extracts from 50 ng down to 0.5 pg were tested by both real-time PCR systems (Figure 3).

Primer and probe set worked at 100% PCR efficiency with approximately three additional cycles (3 Ct) for a 10-fold dilution of template. The method allowed amplification until the dilution factor of 10,000 of the template DNA (corresponding to 5 pg of hazelnut) and showed high correlation coefficient (R^2=0.9995) and PCR efficiency (102.6%) (Figure 3C, Table 3). The number of DNA copies was calculated according to the genome size of hazelnut (0.48 pg) retrieved from the Plant DNA C-values database.[30] This database compiles relevant information regarding several plant species such as genome size (C-value), estimation method, ploidy level, chromosome number and original references. The reference value of 0.48 pg for hazelnut genome size is the most widely used in the literature, since it is considered the prime estimate value for this species. Real-time PCR allowed the amplification of 5 pg of hazelnut corresponding to 10 DNA copies, assuming that the targeted sequences are single copy genes (Figure 3A, Table 3). The limit of quantitation (LOQ) is, by definition, the lowest amount of analyte in a sample, which can be reliably quantitated with acceptable level of precision and accuracy.[28,29]

Table 3. Results of Absolute Detection of Hazelnut DNA by Two Different PCR Systems (Real-Time PCR and Nested Real-Time PCR).

Absolute quantity (pg)	Real-time PCR system		Nested Real-time PCR system	
	Ct ± SD [a]	DNA copies [b]	Ct ± SD [a]	DNA copies [b]
0.5	nd [c]	-	24.14 ± 1.46 (12)	1.0
5	35.37 ± 0.50 (8)	10.4	21.21 ± 0.84 (12)	10.4
50	31.84 ± 0.30 (8)	104	17.83 ± 0.21 (12)	104
500	28.60 ± 0.14 (8)	1042	14.65 ± 0.18 (12)	1042
5,000	25.50 ± 0.07 (8)	10,417	11.64 ± 0.12 (12)	10,417
50,000	22.24 ± 0.08 (8)	104,167	7.81 ± 0.23 (12)	104,167
Correlation coefficient (R^2)	0.9995		0.9988	
Slope	-3.2608		-3.2431	
PCR efficiency (%)	102.6		103.4	

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (n=8) and (n=12). [b] Number of hazelnut haploid genome copies (0.48 pg)[30]. [c] nd, not detected.

In this real-time PCR system, the LOQ equaled the LOD, as the lowest amount of DNA target (5 pg) was within the linear range of the calibration curve.

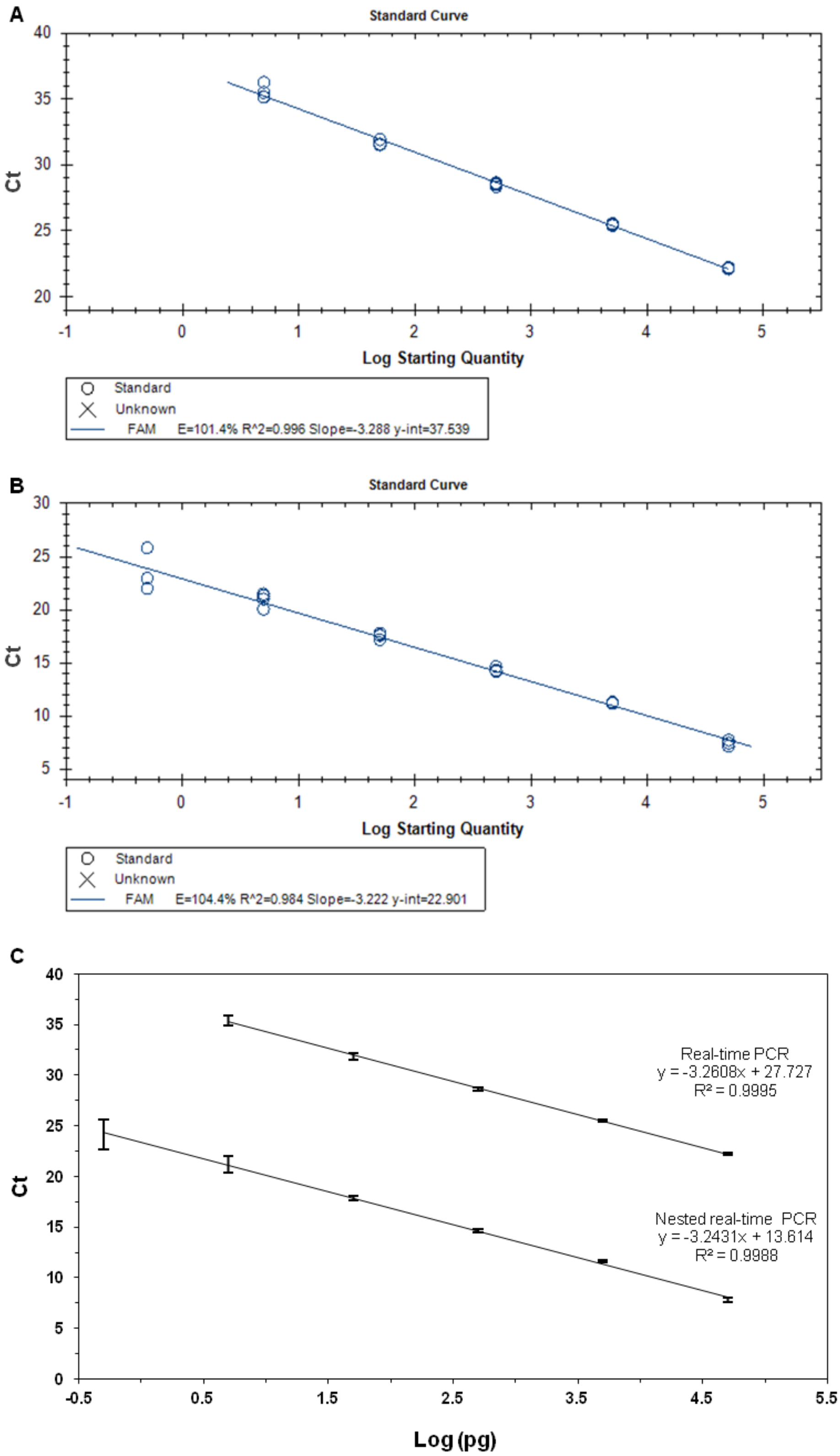


Figure 3. Calibration curves for real-time PCR (A) and nested real-time PCR (B) systems of hazelnut DNA serially diluted (10-fold) from 50 ng to 0.5 pg. Average values and corresponding standard deviations of *n*=8 and *n*=12 replicates for real-time PCR and nested real-time PCR, respectively (C).

Nested real-time PCR system. To evaluate the newly developed single-tube nested real-time PCR system for hazelnut detection, the same set of binary reference mixtures (10% to 0.001%) was used. The protocol defined for this system involved two distinct PCR phases with different number of cycles. In phase 1, the amplification was carried out with outer primers, where 10 cycles were found optimal in terms of highest relative sensitivity at best linearity (data not shown). Using the above conditions, the system permitted establishing a relative LOD of 0.005% (50 mg/kg) of hazelnut in wheat material (Figure 2B, Table 2). In comparison to the initial TaqMan real-time PCR, the former limit of 100 mg/kg was lowered to 50 mg/kg of hazelnut in wheat material. This sensitivity value was also defined regarding the total number of positive replicates in all the performed assays, as it is recommended by the criteria for method performance.[28,29] All assays performed with the nested real-time PCR system presented high correlation coefficient (R^2=0.9956), adequate slope (-3.165) and PCR efficiency (107.0%) (Figure 2C, Table 2).

The absolute sensitivity of the nested real-time PCR system was also assessed with the same range of hazelnut DNA dilutions (50 ng - 0.5 pg) and using 14 cycles in phase 1 of program temperature protocol. The profile of the standard curves for single-tube nested real-time PCR assays evidenced similar behavior to the initial real-time PCR system, with high correlation coefficient (R^2=0.9988), slope of -3.2431 and good PCR efficiency (103.4%) (Figure 2C, Table 3). Single-tube nested real-time PCR assays enabled amplifying 0.5 pg of hazelnut, which corresponds to 1 DNA copy. This absolute LOD for hazelnut was 10× lower in the new method when compared to the above real-time PCR, revealing that this system allows increasing sensitivity by one order of magnitude. The LOQ obtained with nested real-time PCR system was similar to the LOD value, since the lowest amount of diluted hazelnut (0.5 pg) was within the linear range of the calibration curve for all the assays.

Application of nested real-time PCR system. In order to evaluate the application of the developed method to real foods, several chocolate samples and a breakfast cereals sample were tested. The commercial chocolates were carefully chosen to include samples containing hazelnut, other nuts such as almond and also plain chocolate. The results obtained using the single-tube nested real-time PCR system and together with the correspondent label information are presented in Table 4. The food samples were analyzed in parallel amplifications with the reference mixtures containing known amounts of hazelnut for calibration curves. All the single-tube nested real-time PCR assays presented high efficiency (109.5% to 112.9%), slopes ranging from -3.114 to -3.087 and with correlation coefficient of approximately 0.980.

Table 4. Results of the Application of Single-Tube Nested Real-Time PCR to Commercial Food Samples.

Sample	Label Information	Ct ± SD [a]	Estimated Hazelnut (%)
Breakfast cereals with almond s	18% almonds and 8.4% of other crispy nuts (hazelnuts, walnuts, pecan nuts)	19.77 ± 0.28	0.25 ± 0.05
Chocolate crispy nuts	2% hazelnuts, may contain traces of other tree nuts	14.76 ± 0.20	1.95 ± 0.29
Chocolate with hazelnuts and almonds	5% hazelnuts, 5% almonds, may contain traces of other tree nuts	13.84 ± 0.31	3.96 ± 0.89
Black Chocolate	May contain traces of tree nuts	nd [b]	nd
Black chocolate filled with tiramisu	May contain traces of tree nuts	nd	nd
Milk chocolate with almonds	25 % almonds, contain other tree nuts	23.92 ± 0.96	0.013 ± 0.009
Milk chocolate with almonds	15% almond, may contain traces of other tree nuts	nd	nd
Milk chocolate with almonds	11% almonds, may contain other tree nuts	24.95 ± 0.15	0.006 ± 0.001
Milk chocolate with hazelnuts	10% hazelnuts + hazelnut filling (paste), may contain traces of other tree nuts	11.70 ± 0.15	19.6 ± 2.2
Milk chocolate with hazelnuts	12% hazelnuts, may contain traces of other tree nuts	15.22 ± 0.13	7.27 ± 0.72
Milk chocolate with hazelnuts	27% hazelnuts	13.55 ± 0.01	24.4 ± 0.1
White chocolate with hazelnuts	14% hazelnuts, may contain traces of other tree nuts	13.88 ± 0.24	19.6 ± 3.47
Chocolate with hazelnuts	25% hazelnuts, may contain traces of other tree nuts	13.28 ± 0.09	30.4 ± 1.9
Milk chocolate with hazelnut filling	5% hazelnut filling (paste), may contain traces of other tree nuts	13.65 ± 0.19	4.51 ± 0.66
Black chocolate with tree nuts	24.5% of almonds, hazelnuts and raisins, may contain traces of other tree nuts	17.80 ± 0.18	1.09 ± 0.15
Nougat chocolate	7.5% almonds, may contain other tree nuts	24.42 ± 0.57	0.009 ± 0.003
Milk chocolate Fruit & Nuts	7 % almonds, contain other tree nuts	24.02 ± 0.39	0.011 ± 0.003
Truffle chocolate with almonds	8 % almonds, 4% almond filling and hazelnut filling (% not mentioned), may contain traces of other tree nuts	18.20 ± 0.08	0.816 ± 0.005
Chocolate with noisettes	11% hazelnuts, may contain traces of other tree nuts	15.26 ± 0.03	7.16 ± 0.15

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (n=3) of three independent runs. [b] nd, not detected.

In the case of nine chocolate samples containing hazelnut, the results showed that the percentage found for the presence of this nut was always in accordance with the respective label information. The two plain chocolates declaring the information “may contain traces of tree nuts” tested negatively with the proposed nested real-time system. Regarding the potential presence of hazelnut in chocolate samples labeled as “may contain traces of other tree nuts”, four of the chocolates containing known amounts of almond tested positive for hazelnut, while one almond chocolate with the same label information tested negatively. The selection of a certain method over other alternatives is highly dependent on the matrix and/or the allergens present in foods, the required sensitivity and specificity, the time/cost rate, among other factors.

Regarding the protein and the DNA-based methods the choice of the technique also must rely on the availability of good antibody/protein interactions or specific DNA primers/probe and/or on food processing. In the present work, a novel system based on single-tube real-time PCR was developed aiming to achieve higher levels of sensitivity towards the detection of trace amounts of hazelnut in foods, in comparison to the molecular methods available so far. The application of the described nested real-time PCR system as a highly effective molecular approach for the detection of hazelnut encompassed the advantages of the nested PCR and real-time PCR techniques in a single-tube reaction, thus eliminating the problems of cross-contamination frequently associated with the nested PCR. Results from the real-time PCR system using the "outer" primers, i.e. the conventional assay, allowed amplifying 100 mg/kg of hazelnut in wheat material, which is in good agreement with the relative LOD of hazelnut in walnut and hazelnut in flour reported by Piknová et al.[21] and Platteau et al.[18], respectively. The introduction of a new pair of "inner" primers and a different program of temperatures permitted to enhance the sensitivity of the developed method. Therefore, the single-tube nested real-time PCR approach enabled lowering 2× the relative LOD down to 50 mg/kg of hazelnut in wheat material, with 100% of positive replicates in all the performed assays. Regarding the relative LOQ, it was found to be equal to the LOD since the lowest hazelnut standard (0.005%) was within the linear range of the calibration curve, which constitutes an additional advantage on the quantitation of food samples. In terms of absolute LOD, the conventional real-time PCR system tested in this work allowed tracing 5 pg of diluted hazelnut DNA that was lower than the LOD of 9.6 pg and 13 pg of hazelnut reported.[17, 21] The proposed nested real-time PCR system was able to increase the sensitivity by one order of magnitude, allowing the detection of 0.5 pg of hazelnut DNA. To our knowledge, this is the lowest LOD ever reported in the literature for the absolute detection of hazelnut DNA.[18,19,21] Even when compared to the LOD ≤ 5 pg of the commercial kit SureFood allergen Hazelnut (R-Biopharm, Darmstadt, Germany), the single-tube nested real-time PCR presented the best performance regarding the sensitivity level for hazelnut detection. According to the achieved LOD using the nested real-time PCR system, it was possible to detect down to 1 DNA copy of hazelnut.

The optimization of the single-tube nested real-time PCR system enabled detecting hazelnut at trace amounts with high performance criteria and apparent robustness since the system was not affected by additional temperature and time shifts, considering the occurrence of two different reaction protocols within the same real-time PCR run. In all the assays, the parameters were always within the criteria defined by the European Network of GMO Laboratories,[28] which highlights the suitability of the developed method for the identification and quantitation of trace amounts of hazelnut as hidden ingredients in foods.

The successful application of the proposed technique was further demonstrated in real commercial food samples with and without hazelnuts. The estimated percentages of hazelnut in chocolate samples were always in the same order of magnitude as the labeled information, the greater differences being found for samples containing large amounts of hazelnut. It is important to highlight that most of the chocolates contained entire or wrapped hazelnuts, meaning that the values obtained from single chocolate samples (100-200 g) might not be representative of the production lots. Thus, differences found in the semi-quantitation of hazelnut in chocolates are probably due to low size sampling rather than to mislabeling. Regarding the results of two plain chocolates and the sample containing 15% of almond, hazelnut was not detected indicating that the result agreed with the labeled ingredients and that the precautionary labeling was probably unnecessary for nuts/other nuts. However, in the other four almond chocolates with no declared hazelnut, the estimated amounts ranging from 60 mg/kg to 130 mg/kg, justifies the use of precautionary labeling in these cases, which might be the result of cross-contamination during production. The breakfast cereals revealed the presence of 250 mg/kg of hazelnut, which was a rather low value for hazelnut as ingredient, but no further conclusion could be drawn since the label indicated the presence of several crispy nuts, without specifying hazelnut amount.

Taking into account the performance and applicability to the analysis of commercial food samples, the single-tube nested real-time PCR system proved to be a highly specific and sensitive technique for the detection of hazelnut when compared to other methods proposed so far.[17-19,21] However, to validate and correctly implement this and/or other methodologies for monitoring the presence of allergens in foods, it is essential that certified reference materials are developed, such as those in the case of GMO. Official guidelines should also be made available shortly, regulating limits for the presence of potentially allergenic ingredients in food and recommended methodology for their monitoring.

In summary, with this work we were able to present a novel and effective alternative method to detect hazelnut traces in foods, namely in complex food samples such as the case of chocolates. In addition to the simple and highly sensitive real-time PCR assay, we developed for the first time a single-tube nested real-time PCR system as a cost-effective and powerful tool for high-throughput DNA-based identification of hazelnut allergens in foods.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2011, by the project "Improvement of molecular biology methods for authenticity assessment and detection of allergens in foods" (FCT/2489/3/6/2011/S) of Technological and Scientific Cooperation FCT/Slovakia and by University of Porto "Projectos Pluridisciplinares" IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidized by FSE and MCTES).

Supporting Information

Additional table is provided after references.

REFERENCES

(1) Sicherer, S. H.; Sampson, H. A. Food allergy: recent advances in pathophysiology and treatment. *Annu. Rev. Med.* **2009**, *60*, 261-277.

(2) Costa, J.; Mafra, I.; Carrapatoso, I.; Oliveira, M. B. P. P. Almond allergens: molecular characterization, detection, and clinical relevance. *J. Agric. Food Chem.* **2012**, *60*, 1337-1349.

(3) Sicherer, S. H.; Muñoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J. Allergy Clin. Immunol.* **2003**, *112*, 1203-1207.

(4) Wensing, M.; Akkerdaas, J. H.; van Leeuwen, W. A.; Stapel, S. O.; Bruijnzeel-Koomen, C. A. F. M.; Aalberse, R. C.; Bast, B. J. E. G.; Knulst, A. C.; van Ree, R. IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance. *J. Allergy Clin. Immunol.* **2002**, *110*, 435-442.

(5) Bock, S. A.; Muñoz-Furlong, A.; Sampson, H. A. Further fatalities caused by anaphylactic reactions to food, 2001-2006. *J. Allergy Clin. Immunol.* **2007**, *119*, 1016-1018.

(6) Burney, P.; Summers, C.; Chinn, S.; Hooper, R.; Van Ree, R.; Lidholm, J. Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy* **2010**, *65*, 1182-1188.

(7) Holck, A.; Diaz-Amigo, C.; Kerbach, S.; Popping, B.; Mustorp, S.; Axelsson, C. E. Detection of allergens in food. In *Current topics on food authentication*; Oliveira, M. B. P. P., Mafra, I., Amaral, J. S., Eds.; Transworld Research Network: Kerala, India, 2011; pp. 173-210.

(8) Platteau, C.; De Loose, M.; De Meulenaer, B.; Taverniers, I. Quantitative detection of hazelnut (*Corylus avellana*) in cookies: ELISA versus real-time PCR. *J. Agric. Food Chem.* **2011**, *59*, 11395-11402.

(9) Trashin, S.A.; Cucu, T.; Devreese, B.; Adriaens, A.; De Meulenaer, B. Development of a highly sensitive and robust Cor a 9 specific enzyme-linked immunosorbent assay for the detection of hazelnut traces. *Anal. Chim. Acta* **2011**, *708*, 116-122.

(10) Cucu, T.; Platteau, C.; Taverniers, I.; Devreese, B.; de Loose, M.; de Meulenaer, B. ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Addit. Contam. Part A-Chem.* **2011**, *28*, 1-10

(11) Garber, E.; Perry, J. Detection of hazelnuts and almonds using commercial ELISA test kits. *Anal. Bioanal. Chem.* **2010**, *396*, 1939-1945.

(12) van Hengel, A. J. Declaration of allergens on the label of food products purchased on the European market. *Trends Food Sci. Technol.* **2007**, *18*, 96-100.

(13) Ansari, P.; Stoppacher, N.; Baumgartner, S. Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts. *Anal. Bioanal. Chem.* **2012**, *402*, 2607-2615.

(14) Heick, J.; Fischer, M.; Pöpping, B. First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J. Chromatogr. A* **2011**, *1218*, 938-943.

(15) Bignardi, C.; Elviri, L.; Penna, A.; Careri, M.; Mangia, A. Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J. Chromatogr. A* **2010**, *1217*, 7579-7585.

(16) D'Andrea, M.; Coïsson, J. D.; Travaglia, F.; Garino, C.; Arlorio, M. Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (*Corylus avellana* L.) in foods through calibration via plasmid reference standard. *J. Agric. Food Chem.* **2009**, *57*, 11201-11208.

(17) D'Andrea, M.; Coïsson, J. D.; Locatelli, M.; Garino, C.; Cereti, E.; Arlorio, M. Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via real-time PCR. *Food Chem.* **2011**, *124*, 1164-1171.

(18) Platteau, C.; De Loose, M.; De Meulenaer, B; Taverniers, I. Detection of allergenic ingredients using real-time PCR: a case study on hazelnut (*Corylus avellana*) and soy (*Glycine max*). *J. Agric. Food Chem.* **2011**, *59*, 10803-10814.

(19) Pafundo, S.; Gulli, M.; Marmiroli, N. Multiplex real-time PCR using SYBR® GreenER™ for the detection of DNA allergens in food. *Anal. Bioanal. Chem.* **2010**, *396*, 1831-1839.

(20) Köppel, R.; Dvorak, V.; Zimmerli, F.; Breitenmoser, A.; Eugster, A; Waiblinger, H.-U. Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur. Food Res. Technol.* **2010**, *230*, 367-374.

(21) Piknová, L.; Pangallo, D.; Kuchta, T. A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. *Eur. Food Res. Technol.* **2008**, *226*, 1155-1158.

(22) Bergerová, E.; Brežná, B.; Kuchta, T. A novel method with improved sensitivity for the detection of peanuts based upon single-tube nested real-time polymerase chain reaction. *Eur. Food Res. Technol.* **2011**, *232*, 1087-1091.

(23) Brisco, M. J.; Bartley, P. A.; Morley, A. A. Antisense PCR: A simple and robust method for performing nested single-tube PCR. *Anal. Biochem.* **2011**, *409*, 176-182.

(24) Minarovičová, J.; Kaclíková, E.; Krascsenicsová, K.; Siekel, P.; Kuchta, T. A single-tube nested real-time polymerase chain reaction for sensitive contained detection of *Cryptosporidium parvum*. *Lett. Appl. Microbiol.* **2009**, *49*, 568-572.

(25) NCBI, National Center for Biotechnology Information, Bethesda, MD; http://www.ncbi.nlm.nih.gov/tools/primer-blast/ (accessed October 13, 2011).

(26) Binladen, J.; Gilbert, M. T. P.; Campos, P. F.; Willerslev, E. 5′-Tailed sequencing primers improve sequencing quality of PCR products. *Biotechniques* **2007**, *42*, 174-176.

(27) Fajardo, V.; Gonzalez, I.; Martin, I.; Rojas, M.; Hernandez, P. E.; Garcia, T.; Martín, R. Real-time PCR for detection and quantification of red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) in meat mixtures. *Meat Sci.* **2008**, *79*, 289-298.

(28) European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing; European Commission: Brussels, 2008; http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm (accessed March 19, 2012).

(29) Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M. W.; Shipley, G. L.; Vandesompele, J.; Wittwer, C. T. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments *Clin. Chem.* **2009**, *55*, 611-622.

(30) Plant DNA C-values database; http://data.kew.org/cvalues/ (accessed March 19, 2012).

Supporting information. Results of PCR amplifications of *hsp1* gene applied to 18 hazelnut cultivars and several additional plant species (28), including other tree nuts (8).

Name	**Scientific denomination**	**Country of Origin**	**PCR**
18 Hazelnut cv. ("Morell", "Negret", "Grossal", "Buttler", "Ennis", "Pauetet", "Fertile de Coutard"," Sta María del Gesú", "Segorbe", "Tonda di Giffoni", "Culplà", "Merveille de Bollwiller", "Camponica", "Lunga di Spagna", "Cosford", "Gunslebert", "Round du Piémont" and "Lansing")	*Corylus avellana*	Portugal	+
Hazelnut (commercial)	*Corylus avellana*	Portugal	+
Almond	*Prunus dulcis*	Portugal	-
Walnut	*Juglans regia*	France	-
Macadamia nut	*Macadamia tetraphylla*	Austria	-
Pine nut	*Pinus pinea*	Portugal	-
Brazil nut	*Bertholletia excelsa*	Bolivia	-
Pistachio	*Pistacia vera*	USA	-
Cashew	*Anacardium occidentale*	India	-
Chestnut	*Castanea sativa*	Portugal	-
Peanut	*Arachis hypogaea*	USA	-
Lupine	*Lupinus albus*	Portugal	-
Fava bean	*Vicia faba*	Greece	-
Soybean	*Glycine max*	USA	-
Maize	*Zea mays*	USA	-
Wheat	*Triticum aestivum*	Portugal	-
Rice	*Oryza sativa*	Portugal	-
Oat	*Avena sativa*	Portugal	-
Barley	*Hordeum vulgare*	Spain	-
Pumpkins seeds	*Cucurbita mixta*	Greece	-
Rapeseed	*Brassica napus*	Brazil	-
Sunflower	*Helianthus annuus*	Brazil	-
Rye	*Secale cereale*	Portugal	-
Tomato	*Solanum lycopersicum*	Spain	-
Peach	*Prunus persica*	Portugal	-
Apricot	*Prunus armeniaca*	Turkey	-
Plum	*Prunus cerasifera*	Chile	-
Cherry	*Prunus avium*	Chile	-
Strawberry	*Fragaria virginiana*	Spain	-
Raspberry	*Rubus idaeus*	Portugal	-

(Submitted)

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates

Joana Costa[a,b], Parisa Ansari[a], Isabel Mafra[b], M. Beatriz P. P. Oliveira[b], Sabine Baumgartner[a,*]

[a]Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants, Center of Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Austria. [b]REQUI*M*TE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal

*Corresponding author. E-mail address: sabine.baumgartner@boku.ac.at

ABSTRACT

Hazelnut is one of the most appreciated nuts being virtually found in a wide range of processed foods. However, the simple presence of trace amounts of hazelnut in foods can represent a potential risk for eliciting allergic reactions in sensitised individuals. The correct labelling of processed foods is mandatory and the only effective possibility for these patients to avoid eventual adverse reactions. In this sense, adequate methodology for evaluating the presence of offending foods, such as hazelnut, is of great importance. Thus, the aim of this study was to develop a highly specific and sensitive sandwich ELISA for the detection and quantification of hazelnut in complex food matrices. Using model chocolates spiked with known amounts of hazelnut, it was possible to develop an ELISA system capable to detect tracing hazelnut down to 1 mg kg-1 and quantifying this nut down to 50 mg kg-1 in model chocolates. These results highlight and reinforce the value of ELISA as rapid, reliable and cost-effective tools for the detection of allergenic ingredients in foods.

Keywords: immunoassay, hazelnut allergens, Corylus avellana, food allergy, protein-based method.

INTRODUCTION

Hazelnut (*Corylus avellana* L.) is one of the most appreciated edible nuts. This seed is consumed either raw or roasted in snacks and it is used as an ingredient in a wide variety of processed foods (cakes, creams, chocolates and confectionary products) (Alasalvar & Shahidi, 2008). As a consequence, hazelnuts along with the other tree nuts play an important role in economy since they are a significant part of the human food supply (Costa, Mafra, Carrapatoso & Oliveira 2012a). In 2011, among the worldwide production of tree nuts, hazelnuts represented the seventh most relevant culture and occupied the fourth place, in terms of global trade, just behind pistachios, almonds and cashew nuts (FAOSTAT, 2013). Therefore, hazelnuts and other nuts are consumed all over the world by the majority of the individuals in a large variety of forms, which are more or less related to population habits and/or by their geographical availability (Costa et al., 2012a). Although hazelnuts are considered as a safe food, a small percentage of global population can develop adverse immunological reactions after its consumption.

Presently, hazelnut allergy is estimated to have an overall incidence of 7.2%, being this value determined according to a recent multicountry study involving research centres from USA, Australia and eleven countries from Europe (Burney, Summers, Chinn, Hooper, Van Ree, & Lidholm, 2010). Allergy induced by this nut often triggers mild to severe abnormal immunological responses (anaphylactic shocks) that are frequently related to birch pollinosis (Roux, Teuber, & Sathe, 2003). Considering the severity and the frequency of cases involving hazelnut allergy, in addition with the low levels (1 mg of allergenic food) responsible for eliciting observable symptoms upon ingestion (Eller, Hansen, & Bindslev-Jensen, 2012), the sensitised individuals are obliged to completely avoid products susceptible containing hazelnut ingredient.

Thus, in order to safeguard sensitised/allergic individuals, the EU created specific legislation establishing the mandatory labelling of all products containing tree nuts and other ingredients that were included in a list comprising 14 groups of foods considered to be potentially allergenic (Directive 2007/68/EC). To verify the compliance with mandatory labelling, the development of new and more sensitive methodologies for food allergen monitoring is of utmost importance. However, until now no official method was recommended and reference materials are still lacking for the analysis of allergens in foods.

For the detection and quantification of hazelnut in foods, several techniques have been developed, either directly targeting the allergenic proteins (Ansari, Stoppacher, & Baumgartner, 2012; Cucu, Platteau, Taverniers, Devreese, de Loose, & de Meulenaer, 2010; Garber & Perry, 2010; Platteau, De Loose, De Meulenaer, & Taverniers, 2011a;

Trashin, Cucu, Devreese, Adriaens, & De Meulenaer, 2011) or indirectly via DNA analysis (Arlorio, Cereti, Coisson, Travaglia, & Martelli, 2007; Costa, Mafra, Kuchta, & Oliveira 2012b; D'Andrea, Coisson, Locatelli, Garino, Cereti, & Arlorio, 2011, Platteau et al., 2011a,b). In the field of protein-based methods, the enzyme-linked immunosorbent assays (ELISA) are among the most used approaches for allergen detection (Schubert-Ullrich et al., 2009). They are analytical platforms that use antibodies (immunoglobulins) as detecting agents, which specifically bind to the antigen. These tests are highly sensitive, specific and versatile, being able to detect proteins/allergens and other tri-dimensional structures from complex mixtures (Diaz-Amigo, 2010). Immunoassays are more cost-effective than alternative protein mass spectrometry techniques, being adequate for qualitative and quantitative food allergen analysis. However, the development of a reliable ELISA system involves following several parameters: the definition of the target protein(s), the selection of antibodies (monoclonal vs. polyclonal), the choice of animal species for raising the antibodies, the type of ELISA format (competitive vs. non-competitive assays) and the type of labels to be used (Diaz-Amigo, 2010). From all the available immunoassays, the non-competitive sandwich ELISA format is one of the most commonly used. In this format, antibodies are fixed onto a microtiter plate, which specifically bind to the target proteins (allergens). After the recognition of the target molecules/allergens by the fixed antibody, a second antibody is used to attach to this complex. For signal acquisition, a third antibody, usually labelled with an enzyme, a fluorophor or biotin, is normally used. Although ELISA commercial kits have been developed targeting hazelnut allergens or species-specific protein markers, most of them still reveal some issues of cross-reactivity with other species such as sunflower or other tree nuts (walnut and pecan nut).

This work intended to evaluate the adequacy of a sandwich ELISA using anti-hazelnut polyclonal antibody from rabbit and anti-hazelnut monoclonal antibody from mouse to trace and quantify hazelnut from model chocolates. Because of the complexity of the chocolate matrix, the detection of allergens using ELISA systems is usually considered a hard task. In this sense, model chocolates were the elected matrices for the development of the proposed ELISA system. Additionally, it was also aimed at characterising the specificity of anti-hazelnut monoclonal antibodies regarding possible cross-reactivity.

MATERIAL AND METHODS

Reagents and Solutions

For ELISA and immunoblotting analysis the reagents were specific for electrophoresis assays. The water used for the preparation of all buffers and solutions was either purified by reverse osmosis or drawn from a Milli-Q plus system (Millipore, Molsheim, France). Ammonium bicarbonate (≥99%), sodium bicarbonate, sodium carbonate, 2-(N-Morpholino) ethanesulphonic acid (MES, PUFFERAN® 99%), sodium dodecyl sulfate (SDS, ≥99%), 1,4-dithiothreitol (DTT, ≥99%), acetonitrile (HPLC gradient grade), Ficoll-400 and milk powder were purchased from Carl Roth GmbH (Karlsruhe, Germany). Bicine, 3,3',5,5'-tetramethylbenzidine (TMB, 89%), tris[hydroxymethyl] aminomethane (Trizma® base, 99%), ethylenediaminetetraacetic acid (EDTA, 99%), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris, ≥98%), gelatine from cold water fish skin, albumin bovine serum (BSA, fraction V, ≥96%), ammonium acetate (MS grade), hydrogen peroxide (30 wt.%, semiconductor grade), Tween 20, dimethyl sulfoxide (DMSO) and iodoacetamide were acquired from Sigma-Aldrich (Steinheim, Germany). Bis(2-ethylhexyl)sulfosuccinate sodium salt (DONS) was acquired from Fluka Chemie AG (Buchs, Switzerland). Ammonium acetate, ethanol (99% denatured) and sodium azide were obtained from J.T. Baker B.V. (Deventer, Holland). Sodium chloride and citric acid were purchased from Merck (Darmstadt, Germany) and trypsin (sequencing grade modified) from Roche Applied Science (Mannheim, Germany). BCA Protein Assay kit for protein concentration determination was purchased from Thermo Scientific (Rockford, IL, USA). NUPAGE® Novex 4-12% Bis-Tris Gels (1.0-mm thick, 15 well and 1.5-mm thick, 10 well), NUPAGE® LDS sample buffer, Magicmark™ XP western standard 20-220 kDa, SeeBlue® Plus2 prestained standard 4-250 kDa and SimplyBlue™ SafeStain were obtained from Invitrogen Corporation, (Carlsbad, CA, USA). α-Casein and β-casein standards were purchased from Sigma-Aldrich (St Louis, MO, USA).

Preparation of buffers and solutions

For ELISA system, several solutions were previously prepared. The phosphate-buffered saline (PBS, pH 7.5) 0.2 mol L^{-1} was made in 1,000 mL distilled water using 32.22 g of Na_2HPO_4, 2.62 g of NaH_2PO_4 and 21.18 g of NaCl. The PBST1 solution used for washing was done by adding 0.1% of Tween 20 in PBS 0.01 mol L^{-1}. The coating solution (pH 9.6) and the assay buffer (pH 7.5) were prepared by adding 1.22 g of Na_2CO_3, 3.25 g of $NaHCO_3$ and 0.1 g of NaN_3 in 1,000 mL of distilled water and 100 mL of PBS 0.2 mol L^{-1}, 400 µL of Tween 20 in 400 mL of distilled water, respectively. The substrate buffer (pH 4.0) was prepared in 1,000 mL of distilled water by adding 42 g of citric acid and 100 mg of k-sorbate. The substrate solution used in each ELISA was prepared immediately before use, by adding 12.5 mL of substrate buffer (pH 4.0), 2.5 µL of H_2O_2 30% and 100 µL of TMB stock solution (1.6 mmol L^{-1} of TMB, 0.070 mol L^{-1} of DMSO in 25 mL of methanol).

To prepare the NUPAGE® MES SDS running buffer (20×) for SDS-PAGE electrophoresis performance, 60.6 g of Trizma® base, 97.6 g of MES, 10 g of SDS and 3.0 g of EDTA in 500 mL were used. Prior to gel running, the stock solution of NUPAGE® MES SDS was diluted 20-fold with distilled water. For western blot, the Tris-Bicine transfer buffer was made just before performing electrophoresis by adding 4.08 g of bicine, 5.24 g of Bis-Tris, 0.3 g of EDTA and 100 mL of methanol to 900 mL of distilled water. The PBST2 solution used for washing steps during immunoblotting was prepared by adding 0.1% of Tween 20 in PBS 0.2 mol L^{-1}. The substrate solution for it was made with 24 mg of TMB, 80 mg of DONS, 10 mL of ethanol and 30 mL of citrate buffer (0.15 mol L^{-1}, pH 5.0). Immediately before incubation with substrate solution, 5 µL of H_2O_2 was added per each 10 mL of substrate buffer.

Model chocolate preparation

Chocolate with 41% of cocoa and hazelnuts used for model chocolates were purchased at local markets in Portugal. Hazelnuts kernels were grounded to a fine powder in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). Model chocolates containing 50%, 10%, 8%, 5%, 4%, 2.5%, 1%, 0.5%, 0.25%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% of hazelnut were prepared. Chocolate was melted and maintained at 40 ºC during the entire procedure to guarantee correct and complete homogenisation of hazelnut material. The first mixture containing 50% of hazelnut was prepared by adding 100 g of grounded hazelnut to 100 g of melted chocolate. All the following binary mixtures were prepared by serial addition of melted chocolate. The solidified model chocolates were chopped into pieces of approximately 0.3 mm of diameter (Grindomix GM200) and immediately stored at -20 ºC until further analysis.

Other chocolates and hazelnut samples used in this study were purchased at local markets in Austria. A standard reference material of baking chocolate (SRM 2384) acquired from the National Institute of Standards and Technology (Gaithersburg, MD, USA) was also used herein.

Protein extraction

Grounded hazelnut, model hazelnut chocolates and chocolates samples were extracted with two different buffers and in the presence/absence of milk powder (MP) or gelatine from cold water fish (GCF). As these reagents are frequently used to increase the extraction of protein from different matrices, they were added to ensure a better assessment of the proteins present in chocolates. For comparison purposes, samples were also extracted without the addition any of these two reagents. Briefly, 1 g of sample was weighted; with or without the addition of 1 g of milk powder or gelatine from cold water fish to it and 10 mL of 0.2 mol L^{-1} of PBS or 0.1 mol L^{-1} of NH_4HCO_3 were added as the extraction buffers. All mixtures were incubated at 60 ºC for 30 min with occasional mixing. After incubation, they were centrifuged for 15 min (9,400×*g*, 4 ºC) and the supernatant transferred to a new 15 mL falcon tube. If needed, supernatant was further filtrated using a fibre glass filter MN 85/90 BF∅ 45 mm (Macherey-Nagel GmbH & Co., Düren, Germany) and/or a cellulose acetate filter (0.2 µm/pore, Sartorius Stedim Biotech GmbH, Goettingen, Germany) to obtain a clear supernatant. The protein content of extracts was estimated with the commercial BCA protein assay, according to manufacturer's instructions.

ELISA system

The anti-hazelnut antibodies (polyclonal from rabbit and monoclonal from mouse) were produced in-house. The third antibody, anti-mouse-IgG labelled with horseradish peroxidase (anti-mouse-IgG-HRP) used for ELISA and immunoblotting assays were acquired from Sigma-Aldrich (Steinheim, Germany).

Production of anti-hazelnut polyclonal antibodies in rabbit

The polyclonal anti-hazelnut antibodies in rabbit were produced as described earlier (Rudolf et al., 2009). The immunogen mixture was used with a protein concentration of 1 mg mL^{-1} of hazelnut extract.

Production of anti-hazelnut monoclonal antibody

The 6-8 weeks old mice (Balb/c) were immunised with 100 µL immunogen mixture (0.1 mg mL^{-1}) and 100 µL TiterMax. Five subsequent boosters were performed at 2-week intervals with 100 µL adjuvant. After 4 days, a blood sample was taken and screened with indirect competitive ELISA. The last booster was done after 1 week with immunogen mixture (1 mg mL^{-1}) without adjuvant; spleen cell isolation followed 4 days afterwards. Spleen lymphocytes were fused with SP²/0-AG14 mouse myeloma cells (DSMZ, German Collection of Microorganisms and Cell Cultures) using a standard procedure. Further selection and confirmation of monoclonal antibodies were also performed by indirect competitive ELISA. The cells were centrifuged at 400×*g* for 5 min. The supernatant was filtered through 0.45 µm sterile filter (stericcup-HA filter system, Millipore, MA, USA).
The purification of monoclonal antibodies was performed as described by Rudolf et al., (2009) with the affinity chromatographic pre-packed HiTrap Protein G HP columns (Amersham Biosciences, Uppsala, Sweden) and the FPLC system from Pharmacia (Sweden).

Optimisation of ELISA procedure

ELISA was carried out using a non-competitive assay, namely sandwich-type. Each assay was performed in high-binding plates (Greiner bio-one GmbH, Frickenhausen, Germany) coated overnight at 4 ºC with 1 µg mL^{-1} of anti-hazelnut polyclonal antibody from rabbit in coating buffer. Before using, the ELISA plates were blocked with 300 µL of 1% or 2% of Ficoll-400 or 2% of milk powder in coating buffer for 2 h at 37 ºC. Between coating and blocking, the plates were washed once with PBST1 solution. This step was crucial to attain higher homogeneity and reproducibility among wells. The plates were then incubated with the hazelnut standard dilutions and the samples with different concentrations. For checking possible matrix effects, several plates were prepared using hazelnut diluted serially in 5 steps using different blank matrices (commercial chocolate and SRM 2384), which were previously prepared with different extraction buffers and with/without milk powder or gelatine from cold water fish. For testing the model mixtures, a calibration curve was used with hazelnut extract serially diluted in blank matrix (SRM 2384 and chocolate extracted with 0.1 mol L^{-1} of NH_4HCO_3 10-fold diluted) to get 5 different concentrations ranging from 10,000 µg mL^{-1} to 0.13 µg mL^{-1}. The model chocolates were diluted to a concentration that allowed the samples to be read against the linear range of the sigmoid calibration curve. For the evaluation of blank matrices (SRM 2384 and chocolate samples), no sample dilution or 1/10 dilution was used. Protein extracts (100 µL) were incubated in the ELISA plate for 1 h at room temperature with continuous agitation. After incubation, the plate was washed twice with PBST1 solution; then 100 µL of anti-hazelnut monoclonal antibody (1.0 µg mL^{-1}) in assay buffer were added to each well and the plate was again incubated for 1 h with continuous agitation. In order to obtain a colour reaction by the end of the assay, a third antibody marked with an enzyme (HPR) was used, thus after washing the plate once with PBST1, 100 µL of anti-mouse-IgG labelled with HRP (1.0 µg mL^{-1}) in assay buffer were added to each well and incubated for 1 h with continuous agitation. After washing again once with PBST1, the plates were incubated with 100 µL of substrate solution for approximately 2-3 min with continuous agitation in dark conditions, until the formation of a consistent blue colour. The reaction was stopped with 30 µL of 1 mol L^{-1} of H_2SO_4 which lead to the formation of yellow colour in the wells.

Calculations of the calibration curves

The plates were read at 450 nm in a plate reader (Sunrise Remote A-5085, Tecan Group Ltd., Männedorf, Switzerland) and the absorbance results evaluated with Megallan5 software version 5.03 (Tecan Group Ltd., Männedorf, Switzerland). The absorbance values measured at 450 nm were plotted against the logarithmic concentration of the hazelnut protein standard solutions. A non-linear regression function was carried out using a sigmoid four parametric logistic function:

$$B \equiv Y = \frac{A-D}{1+\left(\frac{X}{C}\right)^{b}} + D \qquad (1)$$

where Y is the optical density (absorbance), A the maximum absorbance, b the slope of the calibration curve in linear range, C the 50% inhibition-concentration – IC_{50} (µg L^{-1}), D the minimum absorbance, X the analyte concentration (µg L^{-1}). The calibration curves were further normalised according to the equation:

$$B_{\text{normalised}} = \frac{B}{B_0} \qquad (2)$$

where B is the absorbance of each hazelnut protein dilution and B_0 the absorbance of the highest hazelnut concentration.

Calculation of the recovery

The recovery of the protein from hazelnut was estimated using the concentration values according with the following equation:

$$\text{Recovery (\%)} = \frac{\text{measured hazelnut protein concentration (mg kg}^{-1}\text{)}}{\text{estimated hazelnut protein concentration (mg kg}^{-1}\text{)}} \times 100 \qquad (3)$$

SDS-PAGE, Western-Blot and Immunoblotting

The performance of the anti-hazelnut monoclonal antibody was also checked with western blot. SDS-PAGE was performed on NUPAGE® 4-12% Bis-Tris gels and MES SDS running buffer, as recommended by the manufacturer's instructions, using 2 µg of protein. Electrophoresis under reducing conditions were carried out by adding 50 mmol L^{-1} of DTT during the denaturing heating step, following manufacturer's instructions. The proteins were either visualised with staining the gels using Coomassie (SymplyBlue™ SafeStain) or blotted into a nitrocellulose membrane (western blot). Two different molecular weight markers, namely SeeBlue® Plus2 Prestained standard (4-250 kDa) and Magicmark™ XP western standard (20-220 kDa) were used for staining and blotting, respectively.

To proceed with immunoblotting, at first, the proteins were transferred to a Protran® 0.45 µm nitrocellulose membrane (Schleicher & Schuell Bioscience, Dassel, Germany) using the XCell II™ Blot Module (Invitrogene Corporation, Carlsbad, CA, USA) and Tris-Bicine transfer buffer. The nitrocellulose membranes were blocked with 2% of BSA in 0.05 mol L^{-1} of PBS overnight at 4 ºC. After carefully washing the membrane 3× (10 min each) with PBST2 solution, they were incubated with anti-hazelnut monoclonal antibody (0.05 mg mL^{-1}). The same antibody used in ELISA (anti-mouse-IgG labelled with HRP, 1/1,000 dilution) was also used here. Both incubations were performed for 1 h at room temperature, washing the membranes for 10 min (×3) with PBST2 solution between incubations. The final steps included the addition of 0.01 mol L^{-1} of Tris-HCl (pH 6.0) for 1 min followed by incubation the membrane in substrate buffer for 3-6 min at room temperature and in dark until the development of blue colour. The membranes were then washed with distilled water and scanned. Gels using reduced and non-reduced samples were run.

MS analysis

Protein preparation

After performing SDS-PAGE electrophoresis in reducing and non-reducing conditions, five bands susceptible of containing hazelnut and milk proteins (according to western blot) were cut from the gels and prepared for mass spectrometry (MS) analysis. The gel-bands were placed in 1.5 mL reaction tubes and submitted to proteolysis digestion according to the protocol described for in-gel digestion (http://www.osa.sunysb.edu/Proteomics/ProteinDigestPrep.pdf - last accessed on November 2012) with minor alterations.

Liquid Chromatography – tandem mass spectrometry (LC-MS/MS)

Proteins recovered from electrophoresis gels were submitted to MS analysis using the conditions defined by Ansari, Stoppacher, Rudolf, Schuhmacher, & Baumgartner, (2011) and Ansari et al., 2012. The chromatographic separation was carried out in a 1200 series HPLC (Agilent, Waldbronn, Germany), using an Aquasil C18 reverse phase column (50×2.1 mm, 3 µm particle size, Thermo Electron Corporation, Marietta, GA, USA) and a C18 4×3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). Separation was performed using an injection volume of 5 µL and a flow rate of 0. 5 mL min^{-1}. Two different eluents were used to create a gradient: eluent A (10% of acetonitrile, 5 mmol L^{-1} of ammonium acetate) and eluent B (95% of acetonitrile, 5 mmol L^{-1} of ammonium acetate). HPLC conditions consisted on an initial hold of 0.5 min, linear gradient from 3% to 40% of B within 11.5 min, rapidly up to 100% of B and 4 min hold, switch back to 3% of

B within 2 min and equilibration for 5 min. For MS analysis, a QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) with a Turbo V Ion Spray (ESI) source was used. The optimisation of the selected reaction monitoring (SRM) parameters for profiling of selected tryptic-digested peptides were performed by directly infusing of peptide standard solutions into the mass spectrometer at a flow rate of 10 µL min^{-1}. Analysis of chromatographic and (tandem) mass spectrometric data was carried out using the Analyst™ software version 1.5.2. The ESI source parameters used were defined for positive ionisation mode; curtain gas, 20 psi; both ion source gas 1 and 2, 50 psi; source temperature, 150 ºC for direct infusion and 535 ºC for LC-MS/MS; and +4,000 V of ion spray voltage. The collision gas (nitrogen) was defined to high. For each SRM transition, a dwell time of 40 ms was set.

RESULTS AND DISCUSSION

ELISA procedure

The development of ELISA systems for the specific detection of allergenic ingredients in foods is highly dependent on the selection of proper antibodies, which should present high affinity towards the target protein to ensure better sensitivity (Cucu, Devreese, Trashin, Kerkaert, Rogge, & De Meulenaer, 2012). Although animals can be immunised against almost any molecule, large molecules such as proteins are considered good immunogens (Diaz-Amigo, 2010). In this study, for the specific development of the non-competitive sandwich ELISA for detection and quantification of hazelnut, two sets of antibodies, poly- and monoclonal, were produced against whole hazelnut proteins using rabbit and mouse, respectively. These antibodies were of high-quality, enabling the development of an ELISA system with adequate sensitivity and specificity to detect and quantify hazelnut in processed foods, namely in binary mixtures of chocolates. Considerable contents of substances like polyphenols, saturated and monounsaturated fatty acids, carbohydrates and aromatic compounds are present in chocolate, being regarded as a very complex and difficult matrix to analyse, independently on the type of assay selected (Costa, Melo, Santos, Oliveira, & Mafra, submitted).

In the first experimental attempts for the development of the ELISA system applied to model chocolates containing 50% down to 0.0001% of hazelnut, some inconsistencies were verified. Spiked chocolates with low amounts of hazelnut (≤ 50 mg/kg) and without hazelnut, which were used undiluted or with small dilution (5- or 10-fold), presented high values of absorbance (data not shown). This result was not expected since previous evaluation confirmed the absence of hazelnut in the commercial chocolate by DNA-based methods (Costa et al., submitted). These preliminary results suggested that the anti-hazelnut antibodies could be reacting with other chocolate component. Since the antibodies are raised in animals, their reactivity can be greatly affected by several factors, being quite frequent to cross-react with more than one food component (Cucu et al., 2012).

Matrix effects

To evaluate possible matrix effects of chocolate towards the ELISA system, chocolate without hazelnut (blank chocolate - BC) and a standard reference material of baking chocolate (SRM 2384) were tested. Both chocolate extracts were spiked with hazelnut extracts 5-fold serially diluted in the range of the calibration curve (10,000-0.13µg mL^{-1}). The chocolate extracts were tested undiluted and with a 10-fold dilution (Fig. 1).

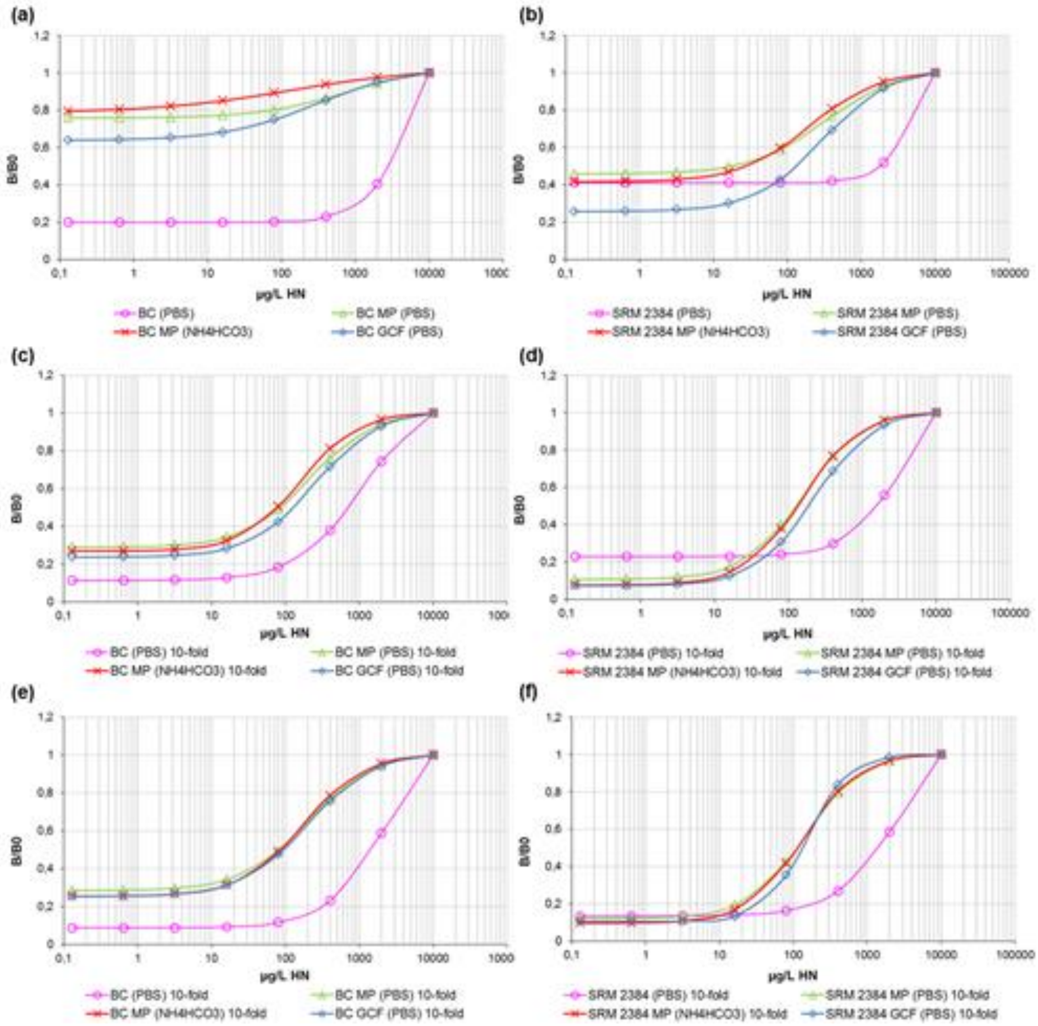


Fig. 1. Normalised curves obtained for chocolate without hazelnut (blank chocolate - BC) (a, c, e) and standard reference material of baking chocolate (SRM 2384) (b, d, f) of undiluted (a, b) and 10-fold diluted (c-d) matrices in ELISA system, using anti-hazelnut polyclonal and monoclonal antibodies. Dynamic range of the calibration curve is 0.13 to 10,000 µg/L). Plate blocking solution used 1% of Ficoll-400 (a-d) or 2% of milk powder (e-f).

For this assessment, the chocolates (BC and SRM 2384) were extracted with two different buffers, 0.2 mol L^{-1} of PBS or 0.1 mol L^{-1} of NH_4HCO_3, in the presence/absence of milk powder or gelatine from cold water fish.

Accordingly, several conditions were defined considering the best performance of the ELISA system to detect hazelnut in chocolate: (i) blocking the plates with 2% of milk powder solution, (ii) washing the plates twice after the incubation of testing samples and (iii) diluting the test samples in blank chocolate, in order to reduce matrix effects.

The influence of the interfering compounds in ELISA was not expected since the monoclonal antibody should only recognise hazelnut proteins. The previously developed anti-hazelnut monoclonal antibody (D9) exhibited some cross-reactivity between proteins from hazelnut and other nuts such as pecan nut, pistachio, cashew, Brazil nut, macadamia and almond. However, the anti-hazelnut monoclonal antibody used in this work belongs to a novel lineage (D10) of antibodies, which is expected to exhibit less cross-reactivity with the other plant species.

Limit of detection (LOD) and limit of quantification (LOQ)

Model chocolates with different contents of hazelnut were serially diluted to establish the LOD. The model mixture containing 50% of hazelnut produced measurable signal until a dilution factor of 100,000. The system enables the detection of 4 ng mL^{-1} of hazelnut protein in assay buffer, considering that for each 1,000 mg of sample (50% hazelnut chocolate) approximately 80 mg correspond to hazelnut proteins, which gives a conversation factor of 12.5. The criteria for the measurable signal was defined taking into account the linear range of the calibration curve, although higher dilution values for hazelnut could be performed. The model chocolate spiked with 0.0005% of hazelnut producing the same signal was obtained with a 10-fold dilution, thus confirming the detection of 4 ng mL^{-1} of hazelnut in assay buffer with the proposed ELISA system. This value is in good accordance with the LOD of 4 ng mL^{-1} of hazelnut protein in phosphate buffer reported by Trashin et al. (2011).

Applicability of ELISA system to model chocolates

All the model chocolates spiked with known amounts of hazelnut were evaluated with ELISA system using the adequate dilutions, according to the linear range of the calibration curve. For this purpose, all model chocolate dilutions were performed using blank chocolate as assay buffer. The ELISA results concerning the estimation of the total amount of protein, hazelnut protein and the percentage of hazelnut, calculated as the relative amount of hazelnut protein in the model chocolates are presented in Table 1.

Table 1 Quantity of hazelnut (%) determined in the model chocolates spiked with known amounts of hazelnut estimated according to ELISA determinations (mg of hazelnut protein/kg of chocolate) and the total extracted protein (mg kg^{-1}).

Model chocolates spiked with hazelnut	Total protein extracted (mg kg^{-1})	ELISA determinations (mg of hazelnut protein/kg of chocolate) ± SD [a]	Estimated amount of hazelnut (%) ± SD	Bias (%)
50%	159746	81350 ± 6881	51 ± 4.3	1.8
10%	164853	15697 ± 756.4	9.5 ± 0.4	4.8
8.0%	135688	11330 ± 1578	8.3 ± 1.1	4.4
5.0%	150966	7917 ± 1095	5.2 ± 0.7	4.9
4.0%	140957	6271 ± 1444	4.4 ± 1.0	11.2
2.5%	150572	2911 ± 113	1.9 ± 0.1	22.7
1.0%	160932	1335 ± 123	0.83 ± 0.1	17.1
0.50%	136750	644.4 ± 294	0.52 ± 0.2	5.7
0.25%	147983	399.0 ± 15	0.27 ± 0.01	7.8
0.10%	171898	176.8 ± 11	0.10 ± 0.01	2.9
0.050%	144307	76.55 ± 2.9	0.053 ± 0.002	6.1
0.010%	180925	15.74 ± 0.31	0.0087 ± 0.0002	13.0
0.0050%	151530	7.19 ± 1.84	0.0047 ± 0.0012	5.1
0.0010%	148753	4.80 ± 0.61	0.0032 ± 0.0004	223
0.00050%	151283	3.68 ± 0.53	0.0024 ± 0.0004	387
0.00010%	151293	2.90 ± 0.46	0.0019 ± 0.0003	1820

[a] mean and standard deviation (SD) of the estimated value of hazelnut in chocolates for *n=12* replicates performed in three independent assays.

Comparing the true values with the estimated relative contents of hazelnut, it can be noted that they were similar. The calculation of bias enabled to verify that all samples until 0.005% of hazelnut in chocolate were within the suitable criteria of acceptance (≤20%). Thus, below that proportion, the estimated ELISA values can only be interpreted for qualitative purposes since the relative errors for the proportions ≤0.0010% were found to be very high. In the case of the three lowest concentrations of hazelnut, extracts were used undiluted or with a 5-fold dilution leading to a higher interference of the matrix and subsequently to the inaccurate quantification of hazelnut in those model chocolates. According to this, the ELISA system is considered an appropriate and reliable tool for the quantification of hazelnut proteins in chocolate down to 50 mg kg^{-1}, while below that value the positive results can only be faced as qualitative information. The method allowed detecting 1 mg kg^{-1} of hazelnut proteins in chocolates, which is in good accordance with the ELISA system developed by Rejeb, Abbott, Davies, Cléroux & Delahaut (2005) to trace hazelnut and other nuts in chocolates. The results presented herein are also in good agreement with those proposed by Holzhauser, Stephan & Vieths (2002) (1 mg kg^{-1} of hazelnut protein) in processed foods like chocolates, cookies, biscuits and breakfast/bar cereals.

In order to access the accuracy and the repeatability of the method, three independent ELISA tests were performed, using four replicates of each protein extract. Mean recovery and the relative repeatability standard deviation (RSD_r) were calculated for the three assays and are represented on Table 2. According to the criteria defined in the validation of a quantitative protein-based method (CAC/GL 74-2010), recovery should be between 70-120%. The mean recovery calculated for the spiking samples of chocolates ranged from 77.3% to 111.2%, which is within the criteria of acceptance for the repeatability of the method.

Table 2 Recovery (%) of hazelnut in model chocolates

Model chocolates spiked with hazelnut	Recovery (%)			Mean recovery (%) [a]	RSD_r (%) [b]
	Assay 1	Assay 2	Assay 3		
50%	111.8	97.6	96.2	101.8	8.5
10%	100.5	92.5	92.6	95.2	4.8
8.0%	120.1	101.5	91.5	104.4	13.9
5.0%	121.3	99.6	93.7	104.9	13.8
4.0%	140.0	91.0	102.6	111.2	23.0
2.5%	73.9	78.8	79.3	77.3	3.9
1.0%	80.0	91.7	77.1	82.9	9.3
0.50%	126.7	73.1	65.9	88.5	37.5
0.25%	103.7	108.0	111.8	107.8	3.8
0.10%	110.0	97.3	101.3	102.9	6.3
0.050%	110.4	102.4	105.6	106.1	3.8
0.010%	85.6	88.9	86.5	87.0	2.0
0.0050%	115.1	67.9	101.7	94.9	25.7
0.0010%	<LOQ [a]	<LOQ	<LOQ	-	-
0.00050%	<LOQ	<LOQ	<LOQ	-	-
0.00010%	<LOQ	<LOQ	<LOQ	-	-

[a] mean recovery (%) and [b] relative repeatability standard deviation (RSD_r) of the estimated value of hazelnut in chocolates for *n=12* replicates performed in three independent assays.

Immunoblotting analysis

An important part of protein analysis is based on the gel electrophoresis since it constitutes a confirmatory tool for the separation and identification of proteins. With separation and gel staining, it is possible to obtain the profiles of protein bands from the extracts (Ansari et al., 2012). After transferring the gel into a nitrocellulose membrane and blocking the free binding sites, the proteins can be detected by the use of specific antibodies (immunoblotting). The application of this technique enables the evaluation of the antibody specificity and the occurrence of cross-reactivity between non-target proteins and the developed antibody. Thus, to assess eventual cross-reactivity related to matrix compounds, several extracts including model chocolates spiked with hazelnut, four different commercial chocolates without hazelnut, milk powder, and proteins from milk (α-casein and β-casein) were tested in this work.

Samples of model chocolates spiked with hazelnut extracted with PBS buffer and milk powder, along with standards (α-casein and β-casein), were primarily run in gel electrophoresis (Fig. 2).

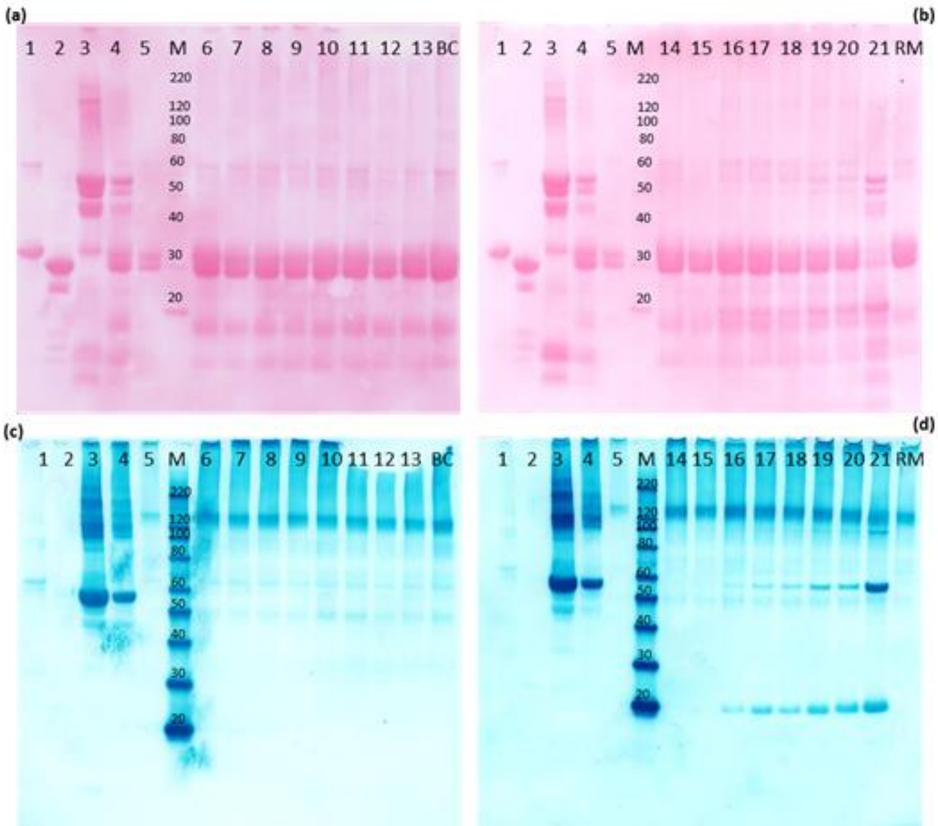


Fig. 2. Western blot membranes stained with Ponceau Red 0.02% for the visualisation of total content of proteins (a,b) and blotted with anti-hazelnut monoclonal antibody (c,d). Chocolate samples were extracted with 0.2 mol L-1 of PBS and 1 g of milk powder. 1, α-casein; 2, β-casein; 3, hazelnut; 4, hazelnut with milk powder; 5, milk powder; 6-21, model chocolates spiked with 0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.25%, 0.5%, 1%, 2.5% 4%, 5%, 8%, 10% and 50% of hazelnut, respectively; BC, blank chocolate used for model chocolate preparation; RM, chocolate standard reference material (SRM 2384); M, molecular weight marker (Magicmark™ XP, Invitrogen).

After staining the western blot membranes with Ponceau Red 0.02%, the protein profiles were visualised (Fig. 2 a,b). Two large sets of bands with molecular weight of approximately 30-40 kDa could be observed in all samples (both model chocolates and hazelnut) that were extracted with milk powder, which seem to correspond to α-casein and β-casein due to their similarity with protein standards and milk powder (Fig. 2 a,b). This profile of bands could not be visualised only on lane 3, hazelnut extracted without milk powder.

The membranes were further incubated with anti-hazelnut monoclonal antibody (Fig. 2 c,d). Model chocolates containing more than 2.5% of hazelnut exhibited a band at approximately 55 kDa with different intensity that could be attributed to the presence of Cor a 9, since it is very close to the molecular mass of 59 kDa defined for 11S globulin-like protein (Beyer, Grishina, Bardina, Grishin, A., & Sampson, 2002; Uniprot, 2013). A second group of bands with ~20 kDa was identified in the same model chocolates. Additionally, a clear band around 150 kDa with the same intensity in milk powder and all chocolates and hazelnut samples extracted with it, was found. This suggests that the specific anti-hazelnut monoclonal antibody bound also to some milk proteins.

Therefore, in order to confirm the results, new gel electrophoresis were run using extracts in reduced (Fig. 3a) and non-reduced (Fig. 3b) conditions. Western blot membranes of those gels were also incubated with anti-hazelnut monoclonal antibody (Fig. 3c,d). Results evidenced the presence of a band at ~30 kDa in reduced conditions, which was not visible with non-reducing conditions. This band is only present in extracts containing chocolate. The gels using reduced and non-reduced proteins were run separately and each gel included two extracts from hazelnut of different batches (2007 and 2012) and extracts from two commercial milk chocolates. Both gels were stained with coomassie blue for the visualisation of the total protein content (Fig. 3a,b). Regarding the extracts ran in reduced and non-reduced conditions, when compared with pure hazelnut, the milk powder presented a very distinct profile of proteins (lanes 1, 3 and 5, Fig. 3a,b). As it was expected, both blank chocolates and the binary mixtures contained the same profile of proteins exhibited in the milk powder, but no protein corresponding to hazelnut profile seems to be present in milk chocolates. This evidence appears to confirm the absence of hazelnut proteins in the blank chocolates tested, including the one used for model mixtures preparation. The apparent binding between milk protein and the specific anti-hazelnut monoclonal antibody could be the explanation for the previous ELISA findings. The easy allowed to confirm the interference of milk in chocolate matrix, but it was investigated more with MS.

LC-MS/MS analysis

For the characterisation of the profile of bands recognised by the anti-hazelnut monoclonal antibody, five sets of bands according to proteins visualised by western blot (one at ca. 150 kDa, three between 49-62kDa and one at ca. 20 kDa) were cut from the gels and submitted to proteolysis digestion to find out, if they really include milk proteins. MS analysis was performed on these in gel digested samples (data not shown) targeting seven marker peptides from milk (β-lactoglobulin, α-lactoalbumin, α-casein and β-casein)

(Ansari et al., 2011) and eight marker peptides for hazelnut (Cor a 8, Cor a 9 and Cor a 11) (Ansari et al., 2012).

The milk peptides either could not be found in these bands, or in very low concentration mainly in the fifth band (20 kDa) and in the samples, which should include milk, which comply with the fact that our target milk proteins are in the range of 14 to 25 kDa. However in the first band (150 kDa), which was suspected to be a milk protein detected by anti-hazelnut antibody, we could not find milk peptides. Low concentration of Cor a 9 could be found in this band only in samples of pure hazelnut and hazelnut with milk powder. An unspecific bound between membrane and antibody could also be the reason for the band visualised by antibody in western blot. The amount of Cor a 9 was high in the third band (ca 55 kDa), which confirm this band as Cor a 9.

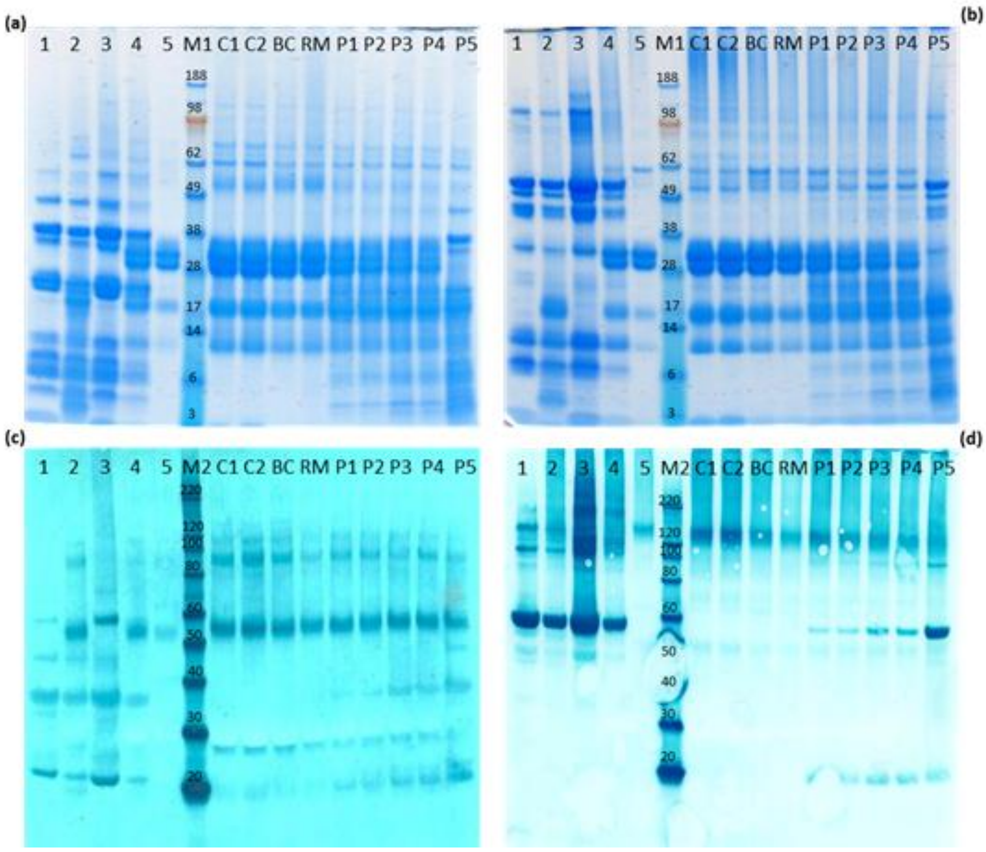


Fig. 3. Comassie blue stained gels for the visualisation of the total content of proteins (a,b) and membranes blotted with monoclonal anti-hazelnut antibody (c,d). Chocolates were extracted with 0.2 mol L-1 of PBS and 1 g of milk powder. (a,c) NUPAGE Novex reduced samples, (b,d) NUPAGE Novex non reduced samples. 1, hazelnut; 2, hazelnut extracted with milk powder; 3, an older extract of hazelnut; 4, older hazelnut extracted with milk powder; 5, milk powder; C1, commercial chocolate 1; C2, commercial chocolate 2; BC, blank chocolate used for model chocolate preparation; RM, chocolate standard reference material (SRM 2384); P1-P5, model chocolates spiked with 4%, 5%, 8%, 10% and 50% of hazelnut; M1, molecular weight marker (SeeBlue Plus2, Invitrogen); M2, molecular weight marker (Magicmark™ XP, Invitrogen).

CONCLUSION

The development of a successful ELISA system for the detection and quantification of hazelnut in foods is difficult to accomplish. Since these systems are highly dependent on the type of antibodies developed, immunological methods such as ELISA have to be carefully evaluated. Herein, we present a sandwich ELISA system with high specificity and sensitivity to detect hazelnut in processed food samples as the case of chocolates. This study also highlighted the importance of using adequate standards for the correct quantification of hazelnut in foods. With this system it was possible to establish a LOD of 1 mg kg^{-1} and a LOQ of 50 mg kg^{-1} for the assessment of hazelnut in complex matrices.

Acknowledgements

The ELISA and MS systems were developed and performed in the Christian Doppler Laboratory “Rapid Test Systems for Allergenic Food Contaminants”, which is funded by the Christian Doppler Research Association and RomerLabs. The authors also thank Marcela Hermann, Institute of Medical Biochemistry, Department of Molecular Genetics, University Vienna, for the immunisation of rabbits as well as Thomas Kolbe, University of Veterinary Medicine Vienna for the immunisation of mice.

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2013 and by University of Porto “Projectos Pluridisciplinares” IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

Alasalvar, C., & Shahidi, F. (2008). Tree Nuts: Composition, Phytochemicals, and Health Effects: an overview. In C. Alasalvar, & F. Shahidi (Eds.), *Tree Nuts: Composition, Phytochemicals, and Health Effects* (pp 1-6). Boca Raton: CRC Press.

Ansari, P., Stoppacher, N., & Baumgartner, S. (2012). Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts. *Analytical and Bioanalytical Chemistry*, *402*, 2607-2615.

Ansari, P., Stoppacher, N., Rudolf, J., Schuhmacher, R., & Baumgartner, S. (2011). Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *399*, 1105-1115.

Arlorio, M., Cereti, E., Coisson, J. D., Travaglia, F., & Martelli, A. (2007). Detection of hazelnut (*Corylus spp.*) in processed foods using real-time PCR. *Food Control*, *18*, 140-148.

Beyer, K., Grishina, G., Bardina, L., Grishin, A., & Sampson, H. A. (2002). Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *Journal of Allergy and Clinical Immunology*, *110*, 517-523.

Burney, P., Summers, C., Chinn, S., Hooper, R., Van Ree, R., & Lidholm, J. (2010). Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy*, *65*, 1182-1188.

CAC/GL 74-2010. Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods, 2010. URL *http://www.codexalimentarius.org/standards/list-of-standards/en/?no_cache=1*. Accessed on 17.10.2013.

Costa, J., Mafra, I., Carrapatoso, I., & Oliveira, M. B. P. P. (2012a). Almond allergens: molecular characterization, detection, and clinical relevance. *Journal of Agricultural and Food Chemistry*, *60*, 1337-1349.

Costa, J., Mafra, I., Kuchta, T., Oliveira, M. B. P. P. (2012b). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry*, *60*, 8103-8110.

Costa, J., Melo, V. S., Santos, C. G., Oliveira, M. B. P. P, & Mafra, I. Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols. *Food Chemistry* (submitted).

Cucu, T., Devreese, B., Trashin, S., Kerkaert, B., Rogge, M., & De Meulenaer, B. (2012). Detection of hazelnut in foods using ELISA: challenges related to the detectability in processed foodstuffs. *Journal of AOAC International*, *95*, 149-156.

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., de Loose, M., & de Meulenaer, B. (2010). ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Additives & Contaminants: Part A*, *28*, 1-10.

D'Andrea, M., Coisson, J. D., Locatelli, M., Garino, C., Cereti, E., & Arlorio, M. (2011). Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via real-time PCR. *Food Chemistry*, *124*, 1164-1171.

Diaz-Amigo, C. (2010). Antibody-based detection methods: from theory to practice. In B. Popping, C. Diaz-Amigo, & K. Hoenicke (Eds.), *Molecular Biological and Immunological Techniques and Applications for Food Chemists* (pp. 223-245). New Jersey: John Wiley & Sons, Inc.

Directive 2007/68/EC (2007). Commission directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients. *Official Journal of European Union*, *L310*, 11-14.

Eller, E., Hansen, T. K., & Bindslev-Jensen, C. (2012). Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. *Annals of Allergy, Asthma & Immunology*, *108*, 332-336.

FAOSTAT. 2013. The Statistics Division of the Food and Agriculture Organization of the United Nations, Rome, Italy. URL http://faostat3.fao.org/home/index.html. Accessed on 04.04.2013.

Garber, E., & Perry, J. (2010). Detection of hazelnuts and almonds using commercial ELISA test kits. *Analytical and Bioanalytical Chemistry*, *396*, 1939-1945.

Holzhauser, T., Stephan, O., & Vieths, S. (2002). Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *Journal of Agricultural and Food Chemistry*, *50*, 5808-5815.

Platteau, C., De Loose, M., De Meulenaer, B., & Taverniers, I. (2011a). Quantitative Detection of hazelnut (*Corylus avellana*) in cookies: ELISA versus real-time PCR. *Journal of Agricultural and Food Chemistry*, *59*, 11395-11402.

Platteau, C. l., De Loose, M., De Meulenaer, B., & Taverniers, I. (2011b). Detection of allergenic ingredients using real-time PCR: A case study on hazelnut (*Corylus avellena*) and soy (*Glycine max*). *Journal of Agricultural and Food Chemistry*, *59*, 10803-10814.

Rejeb, S. B., Abbott, M., Davies, D., Cléroux, C., & Delahaut, P. (2005). Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. *Food Additives and Contaminants*, *22*, 709-715.

Roux, K. H., Teuber, S. S., & Sathe, S. K. (2003). Tree nut allergens. *International Archives of Allergy and Immunology*, *131*, 234-244.

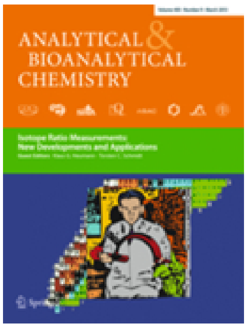
Rudolf J., Führer M., Galler B., Ansari P., Hasenhindl C. & Baumgartner S. (2009). Differences in usability of rabbit IgG and chicken IgY after clean-up and impact on gold labelling properties. *Journal of Immunological Methods*, 350, 79-88.

Schubert-Ullrich, P., Rudolf, J., Ansari, P., Galler, B., Führer, M., Molinelli, A., et al. (2009). Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Analytical and Bioanalytical Chemistry*, *395*, 69-81.

Trashin, S. A., Cucu, T., Devreese, B., Adriaens, A., & De Meulenaer, B. (2011). Development of a highly sensitive and robust Cor a 9 specific enzyme-linked immunosorbent assay for the detection of hazelnut traces. *Analytica Chimica Acta*, *708*, 116-122.

Uniprot (Universal Protein Resource), 2013. URL http://www.uniprot.org/uniprot/Q8W1C2 Accessed on 17.10.2013

(Submitted)



Analytical and Bioanalytical Chemistry

Springer

Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR

Joana Costa[a,b], Parisa Ansari[a], Isabel Mafra[b], M. Beatriz P. P. Oliveira[b], Sabine Baumgartner[a,*]

[a]Christian Doppler Laboratory for Rapid Test Systems for Allergenic Food Contaminants, Center of Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences, Austria. [b]REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal

*Corresponding author. E-mail address: sabine.baumgartner@boku.ac.at

ABSTRACT

Hazelnut (*Corylus avellana* L.) is responsible for a significant part of the allergies related to nuts. Still, is it a very well appreciated nut and as consequence is widely used in all type of processed foods such as chocolates. Correct food labelling is currently the most effective means of preventing the consumption of allergenic ingredients, namely hazelnut by the sensitised/allergic individuals. Thus, to verify labelling compliance and to ensure allergic patient protection, the development of highly sensitive methodologies are of extreme importance. In this study, three major methodologies, namely ELISA, LC-MS/MS and real-time PCR where evaluated for their performance regarding the detection of hazelnut allergens in model chocolates. With sensitivity levels of approximately 1 mg kg^{-1} and limits of quantification of 50-100 mg kg^{-1}, all the performed methods were considered appropriate for the identification of hazelnut in complex foods as chocolates. To our knowledge this was the first successful attempt to develop and compare three independent approaches for the detection of allergens in foods.

Keywords: hazelnut detection, complex foods, model chocolates, DNA-based methods, protein-based techniques, food allergy.

INTRODUCTION

In the last years, food-induced allergies have been regarded with special interest. The number of individuals suffering from some kind of food allergy is not yet well established, although it is generally estimated to affect 1-2% up to 10% of the world's population [1]. Theoretically, any food can be considered as potentially allergenic, however more than 90% of the adverse immunological responses are caused by eight groups of foods, namely milk, egg, fish, crustaceans, tree nuts, peanut, soybean and wheat. As consequence, these foods are usually the focus of several studies, once they are also accountable for most of the severe and potentially life-threatening allergic reactions.

Included in the tree nut group, hazelnut (*Corylus avellana* L.) is responsible for a significant part of the allergies related to nuts. Often associated with birch pollinosis [2] and with an overall incidence of 7.2% [3], allergy induced by hazelnut is one of the nut allergies most well studied. According to the WHO/IUIS list of allergens [4] and to the Allergome database [5], eleven groups of allergenic proteins have been identified and characterised, namely Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12, Cor a 13, Cor a 14 and Cor a TLP (as food allergens) and Cor a 6, Cor a 10 (as pollen allergens), which have been extensively reviewed by Costa et al. [6]. With so many different allergens belonging to a large spectrum of protein families, clinical symptoms associated with hazelnut allergy are known to range from mild (mostly restricted to oral allergy syndrome) to potentially fatal (anaphylaxis). Until now, no effective treatments concerning hazelnut allergy are available, thus, for the sensitised/allergic individuals, the only actual means of preventing allergic reactions consists mainly on the total avoidance of the offending food [6].

Accordingly, the correct labelling of processed foods is of utmost importance for maximising the quality of life of these individuals. In this context, to verify the compliance with labelling and to ensure the protection of such patients, adequate and reliable analytical methods are required for the control of hidden allergenic ingredients in processed foods. In the past years, several protein- and/or DNA-based methods have been advanced for the specific analysis of hazelnut in foods [6]. So far, the methods based on the direct detection of hazelnut allergenic and/or marker proteins have been more extensively applied. Techniques such as the enzyme-linked immunosorbent assays (ELISA) are considered easy to perform with no need for expensive equipment or specialised personnel, therefore those are among the most widely used for the detection and quantification of hazelnut in foods [7-13]. However, the high propensity of the antibodies to cross-react with other plant or animal species constitutes one of the main challenges for developing these protein-based methods. More recently to overcome these drawbacks, the mass spectrometry (MS) platforms have been regarded as excellent tools for the detection of allergenic proteins, namely hazelnut in processed foods [14-16]. In the same sense, but targeting a different biological molecule, the DNA-based techniques have also attained special emphasis for the detection of allergenic foods such as hazelnut. Although using an indirect approach for the assessment of the offending foods, the methods based on the polymerase chain reaction (PCR) have been widely employed for the detection and quantification of this nut especially in processed foods, where the proteins are known to lose their integrity upon processing [17-21]. However, none of the proposed methods have yet been made official for hazelnut or for any other allergenic food. The lack of harmonisation regarding this essential topic continues to contribute to the generalised controversy among researchers and represents key issues in the management of food allergens [6].

In this work, we intended to exploit three different methodologies for the detection of hazelnut in model chocolates. The methods are based on the most representative systems used for the detection of allergens in foods: ELISA, liquid chromatography coupled with mass spectrometry (LC-MS/MS) and real-time PCR. Using the same set of matrices, all systems were developed, tested and compared for their performance regarding their capacity to detect hazelnut in chocolates. All the advantages and drawbacks of each technique were also accounted in this study. To our knowledge, this is the first successful attempt to compare these three systems based on different target-analytes (protein versus DNA) for the assessment of hazelnut allergens in complex food matrices.

MATERIAL AND METHODS

Reagents

All the reagents used for ELISA analysis were of analytical grade. The water used for the preparation of buffers and solutions was either purified by reverse osmosis or drawn from a Milli-Q plus system (Millipore, Molsheim, France). Ammonium bicarbonate (≥99%), sodium bicarbonate, sodium carbonate, 1,4-dithiothreitol (DTT, ≥99%), acetonitrile (HPLC gradient grade) and milk powder were purchased from Carl Roth GmbH (Karlsruhe, Germany). 3,3’,5,5’-tetramethylbenzidine (TMB, 89%), tris[hydroxymethyl] aminomethane (Trizma® base, 99%), ethylenediaminetetraacetic acid (EDTA, 99%), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris, ≥98%), albumin bovine serum (BSA, fraction V, ≥96%), ammonium acetate (MS grade), hydrogen peroxide (30 wt.%, semiconductor grade), Tween 20, dimethyl sulfoxide (DMSO) and iodoacetamide were acquired from Sigma-Aldrich (Steinheim, Germany). Bis(2-ethylhexyl)sulfosuccinate sodium salt (DONS) was acquired from Fluka Chemie AG (Buchs, Switzerland). Ammonium acetate, ethanol (99% denatured) and sodium azide were obtained from J.T. Baker B.V. (Deventer, Holland). Sodium chloride and citric acid were purchased from Merck (Darmstadt, Germany) and trypsin (sequencing grade modified) from Roche Applied Science (Mannheim, Germany). BCA Protein Assay kit for protein content determination was purchased from Thermo Scientific (Rockford, IL, USA).

Model chocolate preparation

Hazelnuts and chocolate with 41% of cocoa used for the preparation of model chocolates were purchased at local markets in Portugal. Hazelnut kernels were grounded to a fine powder in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). Model chocolates spiked with 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% of hazelnut were prepared. Chocolate was melted and maintained at 40 ºC during the entire procedure to guarantee correct and complete homogenisation of hazelnut material.

The first mixture containing 50% of hazelnut was prepared by adding 100 g of grounded hazelnut to 100 g of melted chocolate. All the following binary mixtures were prepared by serial addition of melted chocolate. The solidified model chocolates were chopped into pieces of approximately 0.3 mm of diameter (Grindomix GM200) and immediately stored at -20 ºC until further analysis.

ELISA system

Protein extraction

The extraction of protein from grounded hazelnut and model hazelnut chocolates used in ELISA system was performed according to the described by Costa et al. [22]. Briefly, 1 g of grounded material was weighted out with the addition of 1 g of milk powder. To each mixture, 10 mL of 0.1 mol/L of NH_4HCO_3 were added as the extraction buffer. All mixtures were incubated at 60 ºC for 30 min with occasional mixing

After incubation, they were centrifuged for 15 min (9,400×*g*, 4 ºC) and the supernatant transferred to a new 15 mL falcon tube. If needed, supernatant was further filtrated using a fibre glass filter MN 85/90 BF∅ 45 mm (Macherey-Nagel GmbH & Co., Düren, Germany) and a cellulose acetate filter (0.2 µm/pore, Sartorius Stedim Biotech GmbH, Goettingen, Germany) to obtain a clear supernatant. The protein content of extracts was assessed with the commercial BCA protein assay, according to manufacturer's instructions.

ELISA procedure

The anti-hazelnut antibodies (polyclonal from rabbit and monoclonal from mouse) were produced in-house as described by Costa et al. [22]. The third antibody, anti-mouse-IgG labelled with horseradish peroxidase (anti-mouse-IgG-HRP) used for ELISA was acquired from Sigma-Aldrich (Steinheim, Germany).

ELISA was carried out using a non-competitive assay, namely sandwich-ELISA using the following buffers and solutions: 0.2 mol/L of phosphate-buffered saline (PBS) (0.23 mol/L of Na_2HPO_4, 0.022 mol/L of NaH_2PO_4, 0.36 mol/L of NaCl, pH 7.5); coating buffer (11.5 mmol/L of Na_2CO_3, 39 mmol/L of $NaHCO_3$, 1.5 mmol/L of NaN_3, pH 9.6); washing solution PBST (0.01 mol/L of PBS, 0.1% Tween 20); assay buffer (0.04 mol/L of PBS, 0.08% Tween 20, pH 7.5); substrate buffer (0.22 mol/L of citric acid, 0.67 mmol/L of k-sorbate, pH 4.0) and TMB stock solution (1.6 mmol/L of tetramethylbenzidine, 70 mmol/L of DMSO in 25 mL of methanol).

Each assay was performed in high-binding plates (Greiner bio-one, Frickenhausen, Germany) coated with 1 µg/mL of anti-hazelnut polyclonal antibody (IgG) from rabbit in coating buffer, overnight at 4 ºC. Before blocking the ELISA plates, these were washed once with PBST and then blocked for 2 h at 37 ºC with 2% of milk powder in coating buffer. The plates were then incubated for 1 h at room temperature with the standards (hazelnut serially diluted 1:5, ranging from 10,000 µg/mL to 0.13 µg/mL) for the calibration curve and with the model chocolates diluted in blank chocolate solution to reduce the effects of the matrix. After incubation, the plates were washed twice with PBST solution and then 100 µL of anti-hazelnut monoclonal antibody from mouse (1.0 µg/mL) in assay buffer were added to each well. Plates were again incubated for 1 h with continuous agitation. In order to obtain a colour reaction by the end of the assay, a third antibody conjugated to an enzyme was used. Thus, after washing the plate once with PBST, 100 µL of anti-mouse-IgG labelled with HRP (1.0 µg/mL) in assay buffer were added to each well and incubated for 1 h with continuous agitation. After washing again with PBST, the plates were incubated with 100 µL of substrate solution (substrate buffer (pH 4.0), 0.02% (v/v) of H_2O_2 30% and 0.8% (v/v) of TMB stock solution) for approximately 2-3 min with continuous agitation and in dark conditions, until the formation of a consistent blue colour. The reaction was stopped by adding 30 µL of 1 mol/L of H_2SO_4 with the formation of yellow colour in the wells.

Calculations of the calibration curves and recovery

The calculations that allowed estimating the amount of hazelnut in the model chocolates were also performed according to Costa et al. [22]. Briefly, the plates were read at 450 nm in a plate reader (Sunrise Remote A-5085, Tecan Group Ltd., Männedorf, Switzerland) and the absorbance results evaluated with Megallan5 software version 5.03 (Tecan Group Ltd., Männedorf, Switzerland). The absorbance values measured at 450 nm were plotted against the logarithmic concentration of the hazelnut protein standard solutions. A non-linear regression function was carried out using a sigmoid four parametric logistic function:

$$B \equiv Y = \frac{A-D}{1+\left(\frac{X}{C}\right)^{b}} + D \qquad (1)$$

where Y is the optical density (absorbance), A the maximum absorbance, b the slope of the calibration curve in linear range, C the 50% inhibition-concentration – IC_{50} (µg/L), D the minimum absorbance and X the analyte concentration (µg/L).

The recovery of the protein from hazelnut was estimated using the concentration values according with the following equation:

$$\text{Recovery (\%)} = \frac{\text{measured hazelnut protein concentration (mg kg}^{-1})}{\text{estimated hazelnut protein concentration (mg kg}^{-1})} \times 100 \qquad (2)$$

LC-MS/MS system

Protein extraction and enzymatic digestion

The protein extraction was performed in the same manner like ELISA. 2 mL of each sample extracted with this procedure was transferred to a new reaction tube and completely dried overnight at room temperature in a vacuum centrifuge. The precipitate was then ressuspended in 1000 µL of 0.1 mol/L of NH_4HCO_3, 6 mol/L urea buffer. Protein content in all samples was evaluated with BCA and set to a final concentration of 10 mg/mL of protein. At this stage, the samples were digested according to the in-solution digestion protocol (http://www.osa.sunysb.edu/Proteomics/ProteinDigestPrep.pdf) with minor alterations introduced by Ansari et al. [14] and then analysed by LC-MS/MS.

Liquid Chromatography-Mass Spectrometry

The chromatographic separation of the trypic-digested peptides was carried out as described by Ansari et al. [14] with a 1290 infinity UHPLC (Agilent, Waldbronn, Germany), using an Aquasil C18 reverse phase column (50×2.1 mm, 3 µm particle size, Thermo Electron Corporation, Marietta, GA, USA) and a C18 4×3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). Separation was performed using an injection volume of 5 µL and a flow rate of 0.5 mL/min. The mobile phase consisted of eluent A (10% of acetonitrile, 5 mmol/L of ammonium acetate) and eluent B (95% of acetonitrile, 5 mmol/L of ammonium acetate) used to create a gradient. The LC run started with an initial hold of 0.5 min, linear gradient from 3% to 40% of B within 11.5 min, rapidly up to 100% of B and 4 min hold, switch back to 3% of B within 2 min and equilibration for 5 min.

For MS analysis, a QTrap 4000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) with a Turbo V Ion Spray (ESI) source was used. Chromatographic and (tandem) mass spectrometric data was analysed using the Analyst™ software version 1.5.2. The electrospray ionisation (ESI) source parameters used were defined for positive ionisation mode; curtain gas, 20 psi; both ion source gas 1 and 2, 50 psi; source temperature, 535 ºC; and +4,000 V of ion spray voltage. The collision gas (nitrogen) was defined to high. For each selected reaction monitoring (SRM) transition, a dwell time of 40 ms was set and the pause between mass ranges was defined to 5 ms.

Real-time PCR system

DNA extraction

Based on previous work, the selection of the DNA extraction method was made regarding the best results both by end-point and real-time PCR systems [18, 23]. The selected method for the extraction of DNA from model chocolates was based on the use of Nucleospin Food Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor modifications. Briefly, 200 mg of grounded material were transferred to a 2 mL sterile reaction tube followed by the addition of 700 µL of lysis solution CF pre-heated at 65 ºC and 10 µL of proteinase K (20 mg/mL). After 1 h incubation at 65ºC with continuous stirring, 4 µL of RNase A (10 mg/mL) were added and the mixture incubated for 10 min at 37 ºC with soft stirring. Each mixture was then centrifuged for 10 min (17,000×*g* at 4 ºC) and the supernatant (approximately 550 µL) transferred to a new sterile reaction tube. A second centrifugation step was performed for 5 min in the same conditions and about 450 µL of supernatant were collected to a new sterile reaction tube. To each mixture equal volumes of precipitation solution C4 and ethanol 100% were added. After careful homogenisation by inversion, mixtures were eluted in two steps through one spin column by centrifugation at room temperature (1 min, 13,000×*g*). The column was then washed twice with 400 µL of CQW solution and twice with (700 µL and 200 µL) of C5 solution, with 1 min centrifugations (13,000×*g*) between washings and a 2 min final centrifugation. The DNA was eluted from the column by the addition of 100 µL of CE solution at 70 ºC, incubation for 5 min at room temperature and centrifugation (1 min, 13,000×*g*). Each model hazelnut chocolate was extracted at least twice and the DNA extracts kept at -20 ºC until further analysis. UV spectrometric DNA quantification was carried out on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

Target gene selection

For the specific detection of hazelnut, primers and probe targeting the hsp1 gene, which encodes a low molecular weight heat-shock protein with the same name, was selected from the available literature. Primers HSP1F (AGCGTCGAGAGTGGCAAGTTC) and HSP1R (CCTGCTCGCCTCCGCTTTC) were retrieved from Costa et al. [17] and hydrolysis probe NOCC1P (FAM-CCTGACGATGCGATGCTCGACCAG-BHQ2) was selected from Piknová et al. [24]. The oligonucleotides were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Real-time PCR procedure

Real-time PCR assays were performed in 20 µL of total reaction volume. Each reaction tube comprised 2 µL of DNA (20 ng), 1x of SsoFast Probes Supermix (BioRad, Hercules, CA), 300 nmol/L of each primer (Hsp1F/Hsp1R) and 150 nmol/L of hydrolysis probe Nocc1P. The real-time PCR assays were carried on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (BioRad, Hercules, CA) according to the following temperature protocol: 95 ºC for 5 min, 50 cycles at 95 ºC for 15 s and 66 ºC for 45 s, with the collection of fluorescence signal at the end of each cycle. Data were collected and analysed using the software Bio-Rad CFX Manager 3.0 (BioRad, Hercules, CA). Cycle threshold (Ct) values were calculated using the software at automatic threshold settings. Real-time PCR trials were repeated in three independent runs using four replicates, respectively.

RESULTS AND DISCUSSION

Application of ELISA system to model chocolates

The ELISA system used for the evaluation of model chocolates spiked with known amounts of hazelnut was already previously developed and validated [22]. The estimated results for the relative contents of hazelnut in chocolates are presented in Table 1.

In general, the estimated values for hazelnut in model chocolates were in good agreement with the reference values for each model mixture. With the calculation of bias parameter, it was possible to determine that the samples until the spiked level of 0.005%

(50 mg/kg) of hazelnut in chocolate were within the acceptance criteria (≤25%) [25]. In accordance to this, the limit of quantification (LOQ) set for this system corresponded to the lowest concentration of hazelnut in chocolate (50 mg/kg) with bias below 25%. The model chocolates with the concentration of hazelnut below the LOQ could also be detected with the proposed ELISA system although they can only be interpreted as qualitative information.

Table 1 Application of ELISA to model chocolates spiked with known amounts of hazelnut. Estimation of the quantity of hazelnut (%), bias, recovery and respective relative standard deviation for each model chocolate tested.

Model chocolates spiked with hazelnut	Estimated amount of hazelnut (%) ± SD [a]	Bias (%)	Recovery (%) [b]	RSD_r (%) [c]
50%	52 ± 4.5	4.7	104.7	8.7
10%	9.4 ± 0.5	6.1	93.9	4.8
5.0%	5.3 ± 0.6	6.8	106.8	11.7
1.0%	0.79 ± 0.1	20.7	79.3	12.1
0.50%	0.51 ± 0.2	2.4	98.1	33.8
0.10%	0.10 ± 0.01	1.2	101.2	6.2
0.050%	0.052 ± 0.003	3.9	103.9	5.2
0.010%	0.0086 ± 0.0002	13.9	86.1	2.6
0.0050%	0.0048 ± 0.0010	4.0	96.0	20.8
0.0010%	0.0032 ± 0.0003	222	-	-
0.00050%	0.0024 ± 0.0003	381	-	-
0.00010%	0.0019 ± 0.0003	1815	-	-

[a] mean and standard deviation (SD), [b] mean recovery (%) and [c] relative repeatability standard deviation (RSD_r) of the estimated value of hazelnut in chocolates for *n=16* replicates performed in four independent assays.

In comparison with the other ELISA systems, namely the ones developed by Rejeb et al. [11] and Holzhauser et al. [26], the proposed ELISA also enable to detect down to 1 mg/kg of hazelnut chocolate. The accuracy and the repeatability of the method were ensured by the performance of four independent ELISA, using four replicates of each protein extract. Mean recovery and the relative repeatability standard deviation (RSD_r) were calculated for the four assays and are represented on Table 1. The results of recovery were also in good accordance with the parameters defined for the acceptance criteria of a quantitative protein-based method. Repeatability values ranged from 79.3-106.8% which are within the interval (70-120%) recommended for this type of systems.

LC-MS/MS analysis

Results from the analysis of model chocolates by LC-MS/MS, namely the concentrations of selected hazelnut peptides detected in the digested samples expressed in µg of peptide per kg of chocolate and respective recoveries are presented in Table 2.

Table 2 Results of detection of spiked hazelnut in model chocolates by LC-MS/MS.

Model chocolates spiked with hazelnut	Cor a 801		Cor a 901		Cor a 902		Cor a 903		Cor a 904		Cor a 1101		Cor a 1102		Cor a 1103	
	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)	(µg/kg)	Recovery ± SD (%)
50%	33811.0	102.1±5.6	329886.4	97.0±8.9	278171.9	100.7±8.9	420620.8	92.8±11.1	101928.1	96.3±7.3	20919.3	91.9±12.2	2541.2	81.6±14.0	3345.2	91.1±7.6
10%	2745.1	85.9±16.0	57138.4	99.2±15.7	48094.2	91.5±15.9	76316.2	98.9±17.1	15259.6	85.2±16.4	4833.4	101.5±7.4	709.3	80.7±9.9	937.6	76.1±21.8
5.0%	1212.3	74.9±14.6	34250.3	95.3±6.2	26277.3	93.0±8.5	41496.1	91.3±8.6	7344.2	96.7±7.1	1855.0	94.5±8.0	393.3	65.7±8.6	608.7	78.3±14.5
1.0%	331.7	56.1±6.0	5617.7	100.0±22.9	4782.3	96.9±10.9	7889.7	93.2±15.4	1331.8	94.3±5.0	393.3	92.5±11.5	257.5	60.6±8.6	383.2	69.6±19.2
0.50%	314.3	58.9±12.9	3849.4	90.5±16.4	2998.7	93.1±10.4	4632.5	85.1±13.0	806.5	89.5±11.8	423.8	96.1±7.5	232.4	68.1±20.8	400.6	55.0±3.2
0.10%	275.7	96.2±1.6	779.1	95.5±22.8	743.2	68.6±16.4	977.6	102.0±16.1	195.7	89.114.3	226.7	74.3±19.9	191.9	65.8±24.4	320.0	51.9±2.3
0.050%	259.8	87.3±9.8	478.0	83.5±18.8	565.4	71.5±18.2	658.1	96.1±16.9	83.0	83.0±16.1	222.6	76.4±17.8	178.5	77.1±23.3	284.3	64.2±20.3
0.010%	200.5	91.0±13.3	237.2	82.7±19.4	305.7	86.3±2.1	301.6	76.1±19.1	47.2	79.4±14.4	185.5	74.1±18.0	125.2	88.2±19.4	234.8	50.7±0.7
0.0050%	240.7	92.5±16.0	254.7	96.4±16.7	338.6	60.8±2.1	344.4	73.1±11.6	38.4	57.5±16.8	233.3	85.3±11.3	174.1	56.1±13.1	286.5	60.5±2.5
0.0010%	251.7	91.9±11.1	307.0	103.6±16.6	883.7	84.7±15.0	382.0	73.6±12.4	42.0	81.3±18.1	238.5	90.8±11.9	164.3	78.3±20.9	293.3	50.8±0.5
0.00050%	217.1	95.2±5.7	257.4	84.6±16.3	895.3	93.1±13.7	304.8	77.3±18.9	43.1	72.6±17.3	271.2	98.2±11.9	145.8	65.8±20.3	263.9	53.5±2.0
0.00010%	321.5	97.6±4.3	314.2	85.3±19.9	1372.2	91.5±11.3	461.8	81.4±20.9	48.3	68.3±17.6	238.7	97.4±4.2	218.9	79.9±19.9	337.0	63.3±22.9

Recoveries were calculated using n=3 to n=9 replicates in three independent runs.

Based on previously reported work [14], eight peptides from three hazelnut allergens (Cor a 8, Cor a 9 and Cor a 11) were detected in all the model chocolates. The sequences of the eight peptides (Cor a 801, Cor a 901, Cor a 902, Cor a 903, Cor a 904, Cor a 1101, Cor a 1102 and Cor a 1103) and the performance parameters of LC-MS/MS runs are presented in Table 3.

Table 3 Validation data for the quantitative analysis of hazelnut peptides in model chocolates spiked with known amounts of hazelnut under MS acquisition method.

Allergen	Peptide	MW (Da)	LOD (mg/kg)	LOQ (mg/kg)	Calibration Curve Y = b(±*Sb)* X	Correlation coefficient (R^2)	Linear dynamic range (ng/mL)
Cor a 801	GIAGLNPNLAAGLPGK	1,461.8	1	5000	257 ± 56	0.9987 ± 0.0005	6250-1.25
Cor a 901	ALPDDVLANAFQISR	1,628.9	1	100	201 ± 43	0.9971 ± 0.0006	6250-1.25
Cor a 902	QGQVLTIPQNFAVAK	1,612.9	1	500	212 ± 48	0.9975 ± 0.0022	6250-1.25
Cor a 903	INTVNSNTLPVLR	1,439.8	1	100	211 ± 42	0.9988 ± 0.0010	6250-1.25
Cor a 904	WLQLSAER	1,001.5	1	500	435 ± 64	0.9965 ± 0.0027	6250-1.25
Cor a 1101	AFSWEVLEAALK	1,362.7	1	5000	139 ± 28	0.9973 ± 0.0016	6250-1.25
Cor a 1102	LLSGIENFR	1,047.6	1	5000	784 ± 80	0.9977 ± 0.0013	6250-1.25
Cor a 1103	ELAFNLPSR	1,045.6	1	5000	399 ± 39	0.9965 ± 0.0016	6250-1.25

Figure 1 represents examples of extracted ion chromatograms (XIC) for Cor a 902 peptide of blank chocolate (A), digested dilution of hazelnut (50%) (B) and model chocolate spiked with 50% of hazelnut (C) with a recovery of 92.5%. All the MS runs presented very good performance which is confirmed by the mean correlation coefficients (R^2) above 0.98 as evidenced in Table 3. Recoveries were calculated as a ratio in percentage (%) of model chocolates with hazelnut and added digested nut at the same concentration. In general, the model chocolates evaluated by LC-MS/MS evidenced recoveries within the acceptance criteria (70-120%), with only few exceptions revealing values below this interval (Table 2). The worst recoveries were retrieved from the analysis of peptides Cor a 1102 and Cor a 1103, probably due to the low concentration of these peptides in digested hazelnut, which is in good accordance with the results reported by Ansari et al. [14] during the development of the method.

According to MS analysis, it was possible to detect the eight peptides in all model chocolates until the lowest hazelnut spiking level of 0.0001% (1 mg/kg), however for quantification purposes the LOQ established for the eight peptides was different in each case. Regarding the quantification of the eight peptides from different hazelnut allergens, the highest estimated amounts concerned the peptides from protein Cor a 9. This allergen is considered a major storage protein in hazelnut, which was confirmed by the results obtained in the MS analysis (Table 2). Considering the four peptides from Cor a 9 allergen, the LOQ was lower in the case of peptides Cor a 901 and Cor a 903, corresponding to 0.01% (100 mg/kg) of hazelnut in model chocolates, while for Cor a 902 and Cor a 904 the LOQ was 5× higher (0.05%). Although Cor a 8 and Cor a 11 peptides could also be detected down to the spiked level of 1 mg/kg of hazelnut in model chocolates, the LOQ estimated for those corresponded to 0.5% (5,000 mg/kg) probably as a result of the relative low amount of these peptides in samples.

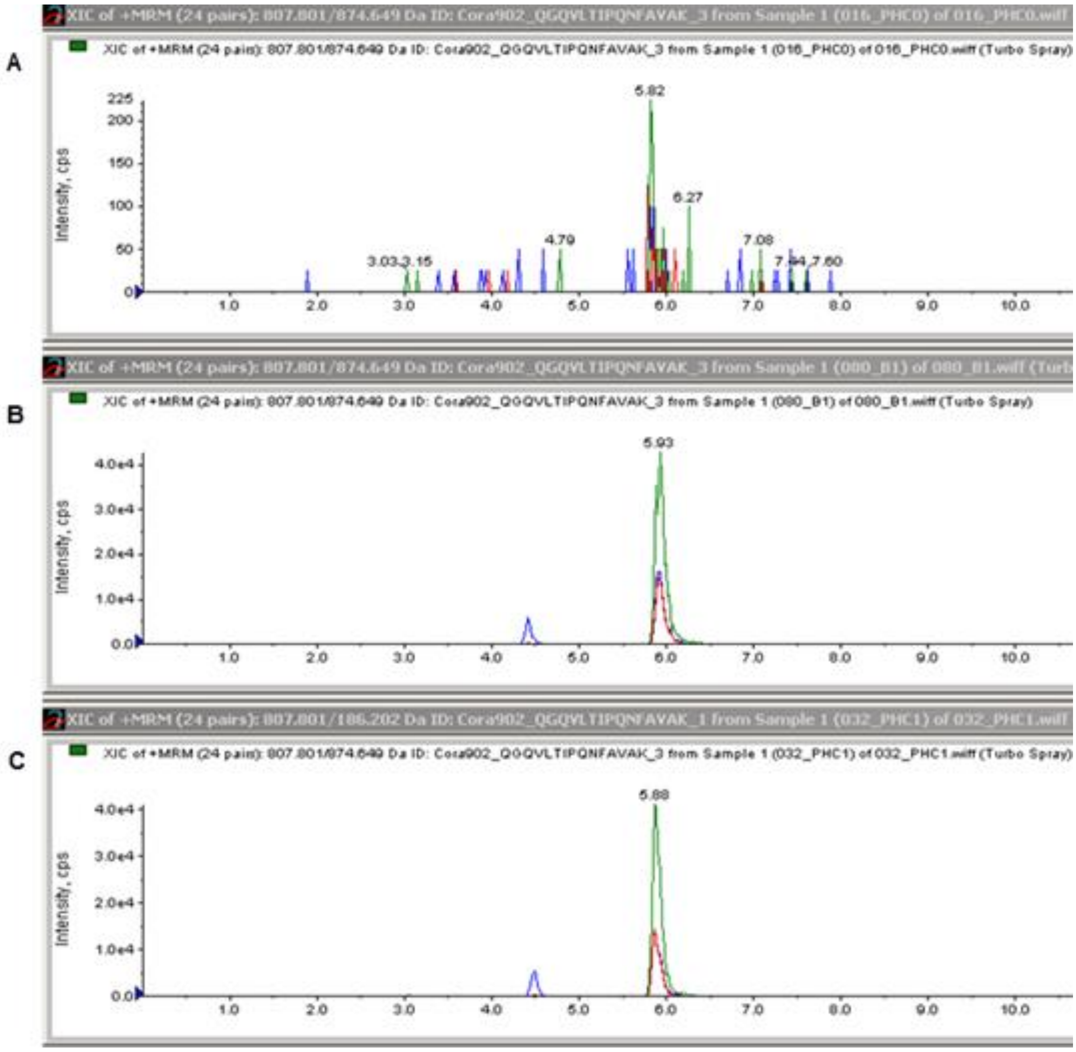


Fig. 1 Extracted-Ion chromatograms (XIC) of peptide Cor a 902 in model chocolate with 50% hazelnut. (A) – chromatogram of blank chocolate (0% hazelnut), (B) – chromatogram of 50% of hazelnut diluted in 0.1 M of NH4HCO3 used for the calculation of recovery and (C) - chromatogram of model chocolate with 50% of hazelnut.

According to these results, the LOD described in this study (1 mg/kg) is comparable to the LOD of 5 mg/kg and 1.3 mg/kg reported by Heick et al. [16] and Bignardi et al. [15], respectively, although the LOQ presented in this study were slightly higher. In addition, eight different marker peptides were used in this study, while in the previous studies only four [16] and two peptides [15] were targeted, which also enable to undoubtedly identify hazelnut in difficult and highly complex matrices such as chocolates.

Application of real-time PCR system to model chocolates

For the evaluation of the developed real-time PCR system several parameters were considered. Since no requirements are yet defined for allergen testing, the prerequisites used to test the real-time PCR were based on the available documents of the definition of minimum performance requirements for analytical methods of genetically modified organism testing [27] and the MIQE guidelines for the minimum information for publication of quantitative real-time PCR experiments [28].

The real-time PCR assays were performed using the already described reference mixtures ranging from 50% down to 0.0001% of hazelnut in chocolate. Figure 2 represents one of the calibration curves for real-time PCR system using model chocolates spiked with 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% of hazelnut.

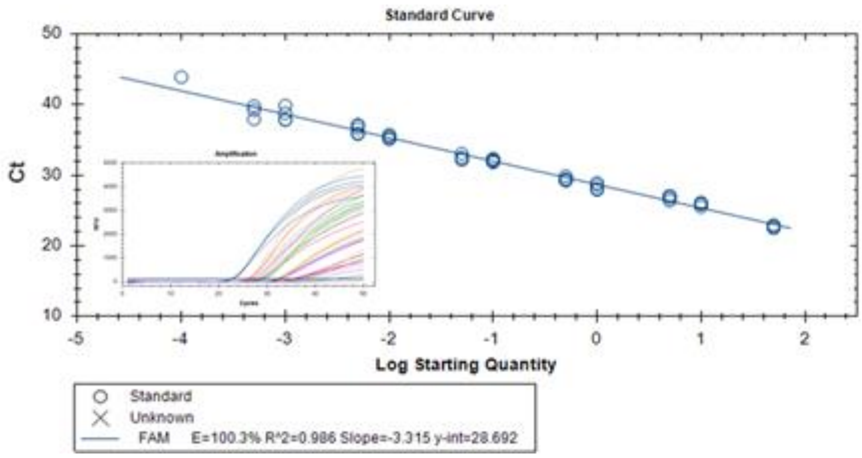


Fig. 2 Example of one of the calibration curves for real-time PCR system applied to model chocolates spiked with 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% of hazelnut. (n=4) replicates per run.

The application of real-time PCR to model chocolates allowed amplifying positively down to the relative level of 1 mg/kg however, the LOD established for the developed system was 50 mg/kg (0.005%) of hazelnut in chocolates (Table 4), which is in good accordance with other research works [17, 218, 23]. According to Mazzara et al. [27] and Bustin et al. [28], the LOD should be defined as the lowest concentration presenting 95% of positive replicates for the target analyte, which in this case was determined accounting with the total number of positive replicates (*n*=12) in all the performed real-time PCR assays. Below the LOD, model chocolates containing 10 mg/kg and 5 mg/kg produced positive amplification in eight out of twelve replicates. The lowest model chocolate spiked with hazelnut (0.0001%) was only positive in 25% of the total replicates.

Table 4 Results of detection of spiked hazelnut in model chocolates by real-time PCR.

Model chocolates spiked with hazelnut	Ct ± SD [a]	CV [b] (%)	DNA copies [c]
50%	22.98 ± 0.07 (12/12)	0.31	20833
10%	25.47 ± 0.15 (12/12)	0.60	4167
5.0%	26.41 ± 0.21 (12/12)	0.81	2083
1.0%	28.21 ± 0.40 (12/12)	1.41	417
0.50%	29.17 ± 0.27 (12/12)	0.93	208
0.10%	31.57 ± 0.27 (12/12)	0.87	41.7
0.050%	31.78 ± 0.68 (12/12)	2.13	20.8
0.010%	35.44 ± 1.54 (12/12)	4.33	4.17
0.0050%	36.69 ± 1.00 (12/12)	2.73	2.08
0.0010%	37.26 ± 1.55 (8/12)	4.16	0.42
0.00050%	38.13 ± 1.93 (8/12)	5.07	0.21
0.00010%	41.23 ± 0.87 (3/12)	2.10	0.042
0%	nd [d] (0/12)	na [e]	na
Correlation coefficient (R^2)	0.9913		
Slope	-3.1376		
PCR efficiency (%)	108.3		

[a] Mean cycle threshold (Ct) values ± standard deviation (SD) (n=12 replicates in three independent real-time PCR runs);
[b] CV, coefficient of variance; [c] Number of hazelnut haploid genome copies (0.48 pg); [d] nd, not detected; [e] na, not applicable.

The LOQ was found to be equal to the LOD (50 mg/kg of hazelnut chocolate) since the lowest amount of hazelnut amplified in the system was within the linear range of the calibration curve. Under the definition of minimum performance requirements, the correlation coefficient (R^2) of standard curves should be above 0.98 and the PCR efficiency between 90% and 110%, implicating slopes ranging from -3.6 and -3.1, respectively [27]. The parameters of the reactions exhibited average values for R^2 of 0.9913, slope of -3.1376 and PCR efficiency of 108.3% (Table 4), being also in good accordance with the acceptance criteria for method performance [27, 28].

The number of DNA copies was calculated according to the genome size of hazelnut (0.48 pg) and assuming that the targeted sequences are single copy genes [29]. Considering the total quantity of DNA (20 ng) used in real-time PCR for each extract of spiked chocolate it was possible to estimate the number of hazelnut DNA copies (Table 4). In all the assays the estimated LOD of 50 mg/kg of hazelnut in chocolate corresponded to a total of approximately 2 amplified DNA copies. Model chocolates spiked with hazelnut below this concentration have less than 1 DNA copy, which could explained the lack of reproducibility in the number of positive replicates, since there is a strong possibility of the target DNA might not be present in all wells. In contrast with the results from ELISA system, the 0% model hazelnut chocolate (100% milk chocolate) failed to amplify in all the replicates and in all the real-time PCR assays. This fact was already expected and further confirms the previous data suggesting that the chocolate used for model chocolates is free from hazelnut residues. When analysing complex samples such as chocolates, the sensitivity values attained in this study are in good accordance with other published works [17, 18, 23].

CONCLUSION

ELISA and more recently MS, are among the most commonly used techniques for the direct detection of allergens in foods. In addition to these two protein-based approaches, real-time PCR is also considered one of the most relevant molecular methodologies applied to the indirect assessment of allergens in foods. Still, the application of the proposed techniques for allergen detection faces different challenges, which might explain the difficulty of officialising one or more methods that fulfil all the crucial criteria.

In this study, the developed ELISA was considered a very reliable method allowing detecting traces of hazelnut proteins down to the spiked level of 1 mg/kg, however the interference of the matrix cannot be neglected. In the case of the analysis of model chocolates containing hazelnut, the application of ELISA system enabled quantifying hazelnut protein until the spiking level of 50 mg/kg with good bias and adequate recoveries. The same results were also attained when applying a real-time PCR system for the detection of hazelnut in model chocolates. Positive amplifications were obtained for the lowest concentration (1 mg/kg) of hazelnut in chocolate. For quantification purposes, the level of 50 mg/kg of hazelnut in chocolate was defined as the LOQ for the proposed real-time PCR system, which according to the hazelnut genome size represent approximately two single copy genes. Using the same set of model chocolates, MS analysis enable detecting hazelnut peptides down to 1 mg/kg, however according to each peptide different values for LOQ were defined (0.01% for Cor a 9 and 0.5% for Cor a 8 or Cor a 11).

Taking in consideration all three methodologies employed for the detection of traces of hazelnut, the performance of those were considered very adequate presenting limits of detection and quantification in the same order of magnitude. These facts evidenced that each of the techniques could be widely applied for the identification of hazelnut in complex matrices such as milk chocolates with similar levels of sensitivity. However, when choosing the technique for allergen detection in foods, some factors have to be considered, such as the availability of expensive equipment and specialised personnel, time consuming per analysis, cost of analysis, among others. For instance, the time needed for sample preparation was higher when applying MS platform (until 3 days), followed by real-time PCR approach (approximately 2 days). In the case of ELISA system, the time for sample preparation is short but in order to set the assay ready to be incubated with samples at least 1 to 2 days were needed. In terms of cost per analysis, MS platform was the most expensive technology employed in this study.

In summary, all the methods presented major advantages and also some drawbacks, still all can be employed with high sensitivity to evaluate the presence of hazelnut in chocolates.

Acknowledgements

The ELISA and MS systems were developed and performed in the Christian Doppler Laboratory “Rapid Test Systems for Allergenic Food Contaminants”, which is funded by the Christian Doppler Research Association and RomerLabs. The authors also thank Marcela Hermann, Institute of Medical Biochemistry, Department of Molecular Genetics, University Vienna, for the immunisation of rabbits as well as Thomas Kolbe, University of Veterinary Medicine Vienna for the immunisation of mice.

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2013 and by University of Porto “Projectos Pluridisciplinares” IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

1. Chafen JJS, Newberry SJ, Riedl MA, Bravata DM, Maglione M, Suttorp MJ, Sundaram V, Paige NM, Towfigh A, Hulley BJ, Shekelle PG (2010) Diagnosing and managing common food allergies: a systematic review. JAMA 303:1848-1856.
2. Roux KH, Teuber SS, Sathe SK (2003) Tree nut allergens. Int Arch Allergy Immunol 131:234-244.
3. Burney P, Summers C, Chinn S, Hooper R, Van Ree R, Lidholm J (2010) Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. Allergy 65:1182-1188.
4. ALLERGEN, (2013). Official site for the systematic allergen nomenclature. http://www.allergen.org/ Accessed 14 March 2013.
5. ALLERGOME, (2013). Allergome database, the platform for allergen knowledge, Latina, Italy. http://www.allergome.org/ Accessed 14 March 2013.
6. Costa J, Mafra I, Carrapatoso I, Oliveira MBPP Hazelnut Allergens: Molecular Characterisation, Detection and Clinical Relevance. Crit Rev Food Sci Nutr (in press).

7. Akkerdaas JH, Wensing M, Knulst AC, Stephan O, Hefle SL, Aalberse RC, van Ree R (2004) A novel approach for the detection of potentially hazardous pepsin stable hazelnut proteins as contaminants in chocolate-based food. J Agric Food Chem 52:7726-7731.
8. Drs E, Baumgartner S, Bremer M, Kemmers-Voncken A, Smits N, Haasnoot W, Banks J, Reece P, Danks C, Tomkies V, Immer U, Schmitt K, Krska R (2004) Detection of hidden hazelnut protein in food by IgY-based indirect competitive enzyme-immunoassay. Anal Chim Acta 520:223-228.
9. Kiening M, Niessner R, Drs E, Baumgartner S, Krska R., Bremer M, Tomkies V, Reece P, Danks C, Immer U, Weller MG (2005) Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. J Agric Food Chem 53:3321-3327.
10. Pele M, Brohee M, Anklam E, van Hengel AJ (2007) Peanut and hazelnut traces in cookies and chocolates: relationship between analytical results and declaration of food allergens on product labels. Food Addit Contam 24:1334-1344.
11. Rejeb SB, Abbott M, Davies D, Cléroux C, Delahaut P (2005) Multi-allergen screening immunoassay for the detection of protein markers of peanut and four tree nuts in chocolate. Food Addit Contam 22:709-715.
12. Röder M, Vieths S, Holzhauser T (2009) Commercial lateral flow devices for rapid detection of peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) cross-contamination in the industrial production of cookies. Anal Bioanal Chem 395:103-109.
13. Trashin SA, Cucu T, Devreese B, Adriaens A, De Meulenaer B (2011) Development of a highly sensitive and robust Cor a 9 specific enzyme-linked immunosorbent assay for the detection of hazelnut traces. Anal Chim Acta, 708:116-122.
14. Ansari P, Stoppacher N, Baumgartner S (2012) Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts. Anal Bioanal Chem 402:2607-2615.
15. Bignardi C, Mattarozzi M, Penna A, Sidoli S, Elviri L, Careri M Mangia A (2013) A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by Liquid Chromatography–Tandem Mass Spectrometry. Food Anal Meth 6:1144-1152.
16. Heick J, Fische, M, Pöpping B (2011) First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. J Chromatogr A 1218:938-943.
17. Costa J, Mafra I, Kuchta T, Oliveira MBPP (2012) Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. J Agric Food Chem 60:8103-8110.
18. Costa J, Oliveira M, Mafra I (2013) Applicability of a real-time PCR system to verify labelling compliance of nut allergens in chocolates. Clin Trans Allergy, 3(Suppl 3):P129.
19. D'Andrea M, Coisson JD, Locatelli M, Garino C, Cereti E, Arlorio M (2011) Validating allergen coding genes (Cor a 1, Cor a 8, Cor a 14) as target sequences for hazelnut detection via real-time PCR. Food Chem 124:1164-1171.
20. Köppel R, Velsen-Zimmerli F, Bucher T (2012) Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. Eur Food Res Technol 235:843-852.

21. Platteau C, De Loose M, De Meulenaer B, Taverniers I (2011) Detection of allergenic ingredients using real-time PCR: A case study on hazelnut (*Corylus avellena*) and soy (*Glycine max*). J Agric Food Chem 59:10803-10814.
22. Costa J, Ansari P, Mafra I, Oliveira MBPP, Baumgartner S. Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates. Food Chem (submitted).
23. Costa J, Melo VS, Santos CG, Oliveira MBPP, Mafra I. Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols. Food Chem (submitted).
24. Piknová L, Pangallo D, Kuchta T (2008) A novel real-time polymerase chain reaction (PCR) method for the detection of hazelnuts in food. Eur Food Res Technol 226:1155-1158.
25. CAC/GL 74-2010 (2010) Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods, Codex Alimentarius International Food Standards, Rome, Italy http://www.codexalimentarius.org/standards/list-of-standards/en/?no_cache=1. Accessed 2 December 2012.
26. Holzhauser T, Vieths S (1999) Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. J Agric Food Chem 47:4209-4218.
27. Mazzara M, Savini C, Charles de Delobel C, Broll H, Damant A, Paoletti C, van den Eede G (2008) European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing, European Commission, Brussels. http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm Accessed 5 July 2013.
28. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611-622.
29. Plant DNA C-values database, Royal Botanic Gardens, Kew, Richmond, Surrey, USA. http://data.kew.org/cvalues/ Accessed 22 July 2013.

CHAPTER 3. WALNUT

STATE-OF-THE-ART

Walnut allergens: molecular characterisation, detection and clinical relevance

Clinical and Experimental Allergy (submitted)

EXPERIMENTAL PART

Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes

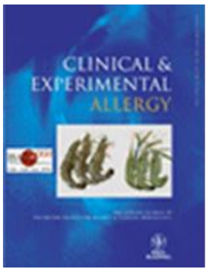
Food Research International, (DOI: 10.1016/j.foodres.2013.09.047**)**

STATE-OF-THE-ART

Walnut allergens: molecular characterisation, detection and clinical relevance

Clinical and Experimental Allergy (submitted)

(*submitted*)



Clinical & Experimental Allergy

Walnut allergens: molecular characterisation, detection and clinical relevance

Joana Costa,[1] Isabel Carrapatoso,[2] Maria Beatriz P.P. Oliveira,[1] Isabel Mafra[*,1]

[1]REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal, [2]Serviço de Imunoalergologia, Centro Hospitalar e Universitário de Coimbra, Portugal

*Corresponding author: Tel: +351 220428640. Fax: +351 226093390. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

Food-induced allergies have been regarded as an emergent problem of public health. Classified as important allergenic ingredients, the presence of walnut and other nuts as hidden allergens in processed foods constitute a risk for sensitised individuals, being a real problem of allergen management. Attending to the increasing importance dedicated to walnut allergy, this review intends to provide the relevant and up-to-date information on main issues such as the prevalence of walnut allergy, the clinical threshold levels, the molecular characterisation of walnut allergens and their clinical relevance, as well as the methodologies for walnut allergen detection in foods. Since the walnut used in human diet comes from *Juglans regia* and *Juglans nigra*, the molecular characterisation of the allergens from both species included in the prolamins (Jug r 1, Jug n 1 and Jug r 3), cupins (Jug r 2, Jug n 2 and Jug r 4) and profilins (Jug r 5), together with respective clinical relevance, were compiled in this review. The most recent progresses on walnut allergen detection techniques (protein- and DNA-based) are described and critically compared, including the emergent multitarget approaches.

Keywords: *Juglans regia*, *Juglans nigra*, food allergens, walnut allergy, prevalence, threshold levels, food oral immunotherapy, clinical relevance, walnut detection.

INTRODUCTION

Walnut belongs to the botanical family of Juglandaceae and it is the seed of the trees of the genus *Juglans*, which encompass 24 different species. The nuts from all the species included in this genus are edible, but only two have economical interest, namely the *Juglans regia* (also designated as common, Persian, English, California, or Carpathian walnut) and the *Juglans nigra* (Eastern black walnut) [1]. Both species are well spread around the globe, being predominantly cultivated in temperate climates. The most well-known species of walnut is the common walnut (*Juglans regia*) that is native from the Balkans region in southeast Europe, southwest and central Asia to the Himalaya and southwest China. The black walnut (*Juglans nigra*) is native from the eastern North America, but also widely cultivated in other regions [1]. There is a large number of walnut cultivars, being Chandler, Hartley, Tulare, Howard, Ashley, Payne, Lara, Franquette, Marbot, Parisienne, Serr, Vina and Valcor, some examples of the varieties originated and/or cultivated in Europe and USA [1, 2].

Like other tree nuts, walnuts have also attained a special interest among world's population since their consumption is frequently associated to healthy habits and equilibrated food diets. The regular consumption of walnut seems to improve the body's lipid profile, contributing to the decrease of cholesterol levels and thus reducing the risk of coronary heart diseases. Additionally, walnuts and other nuts are known to intervene in inflammatory processes, oxidative stress, vascular reactivity and glycemic control (see review [3]), which convert them in excellent foods. The edible part of walnuts are the kernels, though the hulls also present elevated commercial interest, especially those from *Juglans nigra* due to its high pigment (juglone and plumbagin) and tannin content. As consequence, walnut hulls have multiple applications, not only as dyes and inks, but also as potent antibacterial, antimicrobial and antifungal agents [4].

Taking into consideration all the human health benefits and the numerous industrial uses attributed to walnut, the demand for this nut has been increasing, which is supported by the growth of its global production (approximately 270%) during the past 20 years [5]. In 2011, the world's total production of walnut corresponded to 3,418,502 tonnes, ranking this nut in the second place in terms of total production quantity, just behind cashew nut. In the same year, the major walnut producers were China and Iran, accounting for almost 63% of the world's production [5].

The recognised health effects of walnut, combined with its pleasant taste, have led to its inclusion (as seed or oil) in several cookery dishes and pastry products (e.g. cakes, biscuits, ice-creams) [6]. The walnut consumption is frequent and mostly appreciated by the majority of the individuals. However, for a considerable part of the general population the ingestion of walnut can represent a health risk due to the possibility of inducing hypersensitivity in sensitised/allergic individuals. As preventive measure since 1985, the Codex Alimentarius Commission suggested the mandatory labelling of foods susceptible of containing potentially allergenic ingredients. From 1993, walnut and other tree nuts were defined as one of the eight groups accountable for almost 90% of human food allergies [7]. Since then, legislation has been issued and further updated in order to protect sensitised/allergic individuals. Inside the European Union (EU), the food producers are obligated to declare all the ingredients present in pre-packaged foods [8]. Currently, EU legislation establishes a list of fourteen groups of certain substances or products causing allergies or intolerances that are required to be emphasised from the rest of the ingredients enumerated in processed foods, regardless of their quantity [9, 10]. In the referred list, walnut together with other tree nuts, represent one of the fourteen groups of potentially allergenic foods, which also includes soybean, cereals containing gluten, sesame, mustard, celery, peanuts, milk, eggs, fish, molluscs, crustaceans, lupine and sulphites.

Over the past years, tree nuts have been the focus of several studies about food allergies. With this review, it is intended to provide a broad and updated overview about some of the most relevant issues regarding walnut as an allergenic food. Topics such as the prevalence of walnut allergy, the molecular characterisation of identified allergens (from both *Juglans regia* and *Juglans nigra*) and the available analytical methods for its detection will be focused in this review. In addition, other pertinent subjects concerning the clinical relevance of walnut allergy, the definition of threshold levels and an insight on forthcoming potential immunotherapies for food allergies will also be addressed.

GENERAL CONSIDERATIONS ABOUT FOOD ALLERGY

According to the guidelines of National Institute of Allergy and Infectious Diseases in the USA, food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food [11]. Each food is composed by a complex set of proteins that can behave differently regarding to their potential to sensitise and interact with the immune system [12]. In theory, any food can be susceptible of triggering allergic reactions in sensitised individuals, still almost 90% of the total food-induced allergies are restricted to eight groups of foods (milk, egg, fish, crustaceans, soybean, peanut, tree nuts and cereals containing gluten) [11]. Food allergens are biochemically defined as water-soluble glycoproteins with 10-70 kDa of size, presenting high resistance to heat, acid and protease activity [12]. As consequence, allergic reactions can occur when food is ingested as raw or after being cooked or even digested [13].

The adverse immunological responses are classified as immunoglobulin-E (IgE) mediated, non-IgE mediated, or a combination of both [13]. Most of IgE-mediated reactions are featured by a series of symptoms (involving skin, gastrointestinal, cardiovascular and/or respiratory tracts) that commonly appear during the first 2 hours upon ingestion of the offending food. After the allergic sensitisation, food-specific IgE antibodies are produced and bounded on the surface of the mast cells and blood basophils. Upon re-exposure to the given food, the allergenic proteins are recognised by the specific IgE antibodies, which are responsible for activating the release of mediators such as leukotrienes, prostaglandins and histamine [12, 13]. Without observable clinical symptomatology, the sensitisation alone is not sufficient to classify as food allergy [11].

The cell-mediated immunologic reactions (non-IgE-mediated) have different clinical presentations often associated with proctolitis, enterocolitis and enteropathy syndromes that affect primarily infants and young children. In those cases, patients frequently exhibit abdominal cramps, diarrhoea and/or vomiting. Clinical syndromes related to eosinophilic esophagitis and atopic dermatitis are examples of food allergy caused by mixed IgE- and cell-mediated disorders [11, 12]. Abnormal immunological responses can be catalogued in different types, namely immediate and delayed hypersensitivity reactions. The immediate hypersensitivity reactions are mediated by IgE and clinical symptoms ensue generally within 2 hours after ingestion or exposure to the offending food [13]. Despite of being very rare, the appearance of observable symptoms after 24 hours or more upon the ingestion of the allergenic food are classified as delayed hypersensitivity reactions. In these conditions, the adverse responses are cell-mediated and normally activate the T-cells [14]. Although the cell-mediated responses such as allergic contact dermatitis are catalogued as immune-mediated reactions, they are the most uncommon form of food allergy [13]. For this reason, they are classified of small clinical relevance when compared to other immune mediated adverse food reactions.

The severity of the allergic reactions varies from mild to potentially life-threatening and it is dependent on a variety of internal and external factors [15]. Several features have been investigated and are pointed out as possible risks and causes for the development of food allergies. Accordingly, genetic aspects (familial associations and specific genes), association with atopic diseases (e.g. atopic dermatitis) and pre-existing conditions (e.g. asthma), timing and route of exposure to allergen (e.g. topical/respiratory exposure may be sensitising), components of diet (reduced consumption of ω-3 polyunsaturated fatty acids, vitamin D), diet during pregnancy and medication (e.g. antibiotics, antacids) have been highlighted as potential increasing risk factors for food allergy [16]. The severity of the allergic reactions is highly dependent on the amount of food ingested, on the type of food processing used (cooked, raw, or processed) and on the possible interactions with other foods [11]. In addition, factors such as patient's age, speed of food absorption and ingestion of food close to a time of exercise can also contribute to the severity of the allergic reaction. Although all those conditions are known to enhance the severity of an allergic response, its degree of intensity is very difficult to predict. Still, severe symptoms can frequently be observed after mild or severe pre-existing reactions [13].

In the past few years, the knowledge about different aspects of food allergies have been actively used to protect and enhance the quality of life of the sensitised/allergic individuals, still much work is needed in this field.

PREVALENCE OF WALNUT ALLERGY

Food-induced allergy is regarded as an emerging problem of public health affecting adults and children, whose prevalence seems to be rising, not only in the industrialised countries, but also in developing and emerging economies [17]. The true prevalence of food allergies has been very difficult to estimate. Until now, data suggest that 3-4% of adults and 5-6% of young children/adolescents can suffer from some type of allergy related to food [18, 19]. Although the available data is considered rather inaccurate, general numbers estimate that 1-2% up to 10% of the world's population can be affected by food allergy [20].

Most of the studies designed to evaluate the prevalence of food allergies are frequently based on self-reported reactions to foods such as questionnaires and surveys, rather than objective assessments as open food challenges (OFC), double-blind placebo-controlled food challenge tests (DBPCFC), or determined sensitisation to foods by serum IgE and skin prick tests (SPT) [21]. Although questionnaires and surveys could represent helpful tools, respective data must to be carefully interpreted since individuals often tend to overestimate potential symptoms. With respect to some of these facts, the prevalence data is still far from being accurate and should be always regarded as mere indicators of the true incidence of food allergies [22].

Although, some studies have been advanced estimating the prevalence of tree nut allergies [23-27], the information concerning the specific incidence of walnut allergy is still very scarce. Walnut allergy seems to have more expression in the USA together with allergies towards almond and cashew, in opposition to Europe were hazelnut allergy is more frequently common [25, 28].

On the basis of a large Europe-wide research project (EuroPrevall) funded by the European Commission that was specifically elected to evaluate the prevalence, basis and cost of food allergies [29], data about the prevalence of walnut allergy were recently provided. In this project, involving numerous centres from a total of 13 countries (USA, Australia and eleven countries from Europe), 5 allergen mixes from a total of 24 foods previously defined as priorities (including walnut) were tested against the sera of sensitised/allergic subjects. Walnut allergy presented an overall incidence of 2.2% and a prevalence of 1.8% after excluding the birch positive individuals [30]. France exhibited the highest incidence of walnut allergic patients (3.7%), closely followed by Germany (3.3%), Italy (3.1%) and Spain (3.1%). From the total of 24 foods evaluated, walnut allergy ranked the fifteenth position, being the first occupied by hazelnut [30]. This ranking evidenced the relevance of hazelnut allergy in Europe, considering that most of the countries participating in this wide project were European.

With respect to the presented information, it becomes clear that more extensive studies are needed to support the estimated data for the prevalence of walnut allergy. In addition, it would also be very important to include objective information regarding walnut and other nut allergies involving more countries from different regions (e.g. Africa, Asia).

CLINICAL THRESHOLD LEVELS FOR WALNUT

The cure for food allergies is not yet available. Therefore, for the sensitised/allergic individuals the only effective means of preventing an adverse reaction is the total avoidance of the offending food or a therapeutic treatment (antihistaminic, corticosteroids, bronchodilators and/or epinephrine) in the case of an accidental exposure to the allergenic food [15]. Since food-allergic consumers represent a small, but rather significant growing portion of the general population [31], protective measures such as precautionary labelling have been widely adopted by food processing industry to prevent possible legal actions. The use of precautionary labelling safeguards the allergic population, but also restricts their choices when acquiring processed foods. In this sense, the knowledge of the minimum dose that can elicit a reaction is of great interest to all food allergy stakeholders (caretakers, health professionals, food industry) [32, 33]. The definition of clinical thresholds for allergenic foods is fundamental in evaluating the risk from those at both the individual and population levels. While the information about individual thresholds permits allergic patients and health professionals to better manage food allergy, the insight about population thresholds should help food industry and regulatory authorities assessing appropriate guidelines for risk management of allergenic foods [32]. The clinical threshold level for an allergenic food lies between the highest dose observed, not inducing any adverse effect (no observed adverse effect level - NOAEL), and the lowest dose of food triggering an observed adverse effect (lowest observed adverse effect level - LOAEL) [34]. Since this limit is difficult to delineate, threshold is often defined as the LOAEL or the NOAEL on both population and individual basis [34].

When in contact with the offending food, allergic individuals can respond differently to a very large range of doses. As a result, the assessment of individual threshold levels can be determined by clinical challenge trials such as OFC and DBPCFC. Results from those challenges can be extrapolated to provide a dose-response curve to a given allergen by means of statistical models based on individual LOAEL parameters [35]. Nevertheless, these tests must involve the cooperation of the allergic patients during small or long periods, which is not always easily carried out.

The determination of clinical thresholds for allergenic foods is still at a very preliminary stage. In the particular case of the group of tree nuts, only primary data have been advanced for hazelnut, cashew nut and walnut [36, 37]. Regarding walnut, a single study based on DBPCFC was reported by Blom et al. [36] in which the retrieved information was analysed to determine individual LOAEL and NOAEL for both subjective and objective symptoms. The DBPCFC were applied to a total of 363 allergic children/adolescents with ages ranging from 2 to 16 years-old in order to trace a profile of threshold levels for 7 different allergenic foods, namely peanut, egg, milk, cashew nut, hazelnut, soybean and walnut. Statistical models were applied to define population thresholds using LOAEL and NOAEL values expressed as discrete doses in milligram of total protein of the allergenic food [36]. Threshold levels were established for 5 major allergenic foods (cashew nut, hazelnut, egg, milk and peanut). However, the number of individuals with positive objective symptoms for walnut was too small to fit into distribution models and only individual LOAEL and NOAEL were defined for this allergenic ingredient. The positive LOAEL with objective symptoms exhibited a wide scope of doses, ranging from 0.9 to 350 mg of walnut total protein in baked cookies [36].

From the facts exposed, it is well reinforced that much research is still needed in order to establish clear clinical threshold levels for walnut and other allergenic foods such as different nuts.

MOLECULAR CHARACTERISATION OF WALNUT ALLERGENS

Different allergenic proteins have been identified in walnut, concerning the two species *Juglans regia* and *Juglans nigra* since they are both involved in walnut allergy. Therefore, the allergens identified so far, for each of the referred species, were included in this review. Common and black walnut allergens were included in two superfamilies of proteins, namely the prolamin (Jug r 1, Jug n 1 and Jug r 3) and the cupin (Jug r 2, Jug n 2 and Jug r 4). More recently, another allergen (Jug r 5) was identified in walnut as belonging to a different family of proteins, the profilins.

At the time, proteins Jug r 1, Jug r 2, Jug r 3 and Jug r 4 from common walnut and Jug n 1 and Jug n 2 from black walnut were already included and classified as food allergens in the WHO-IUIS list of allergens [38]. The profilin Jug r 5 was not yet included in the referred list, but it was already comprised in the Allergome database [39].

Prolamin superfamily

This superfamily of proteins was initially characterised by its solubility in alcohol-water mixtures, either in its native state or after the reduction of inter-chain disulphide bonds and by its high content in amino acids such as proline and glutamine, thus justifying its designation (prolamin) [40, 41]. It encompasses three major groups of food allergens: the 2S albumins, the non-specific lipid transfer proteins (nsLTP) and the cereal alpha-amylase/trypsin inhibitors. The members of the prolamin superfamily present a highly conserved pattern of cysteine residues located within a sequence of about 100 amino acids (aa). They exhibit six or eight cysteine residues forming three or four intra-chain disulphide bonds, respectively [41]. In general, these proteins have low molecular weight, high cysteine content, similar three-dimensional structures rich in alpha-helices and high stability to thermal processing and proteolysis [42]. Due to characteristics such as high stability to heat and to gastrointestinal digestion, many of the allergens belonging to prolamin superfamily are considered important class I food allergens responsible for inducing severe allergic reactions (e.g. anaphylaxis) in sensitised individuals [43].

2S Albumin family

Included in the prolamin superfamily of proteins, Jug r 1 and Jug n 1 were identified as allergens from common walnut and black walnut, respectively. Sharing the same general characteristics of the prolamin superfamily members, the 2S albumins are considered water soluble proteins at low salt concentrations, with a primary structure presenting high content in arginine, glutamine, asparagine and cysteine residues. After their synthesis, these small globular proteins (12-15 kDa) are subjected to sequence modifications that include the cleavage of 2S albumins into a large and small subunits (heterodimers) linked by conserved inter-chain disulphide bonds [44]. Due to the presence of eight cysteine residues that are distributed in a conserved pattern, the conformational structure of 2S albumins is ensured by four disulphide bonds. During germination, the 2S albumins are thought to act as important nitrogen and sulphur donors, as a result of the amino acid composition of these proteins, their high abundance and their mobilisation in the seed cells [45, 46]. In addition to their active role in the germination of seeds, other functions have also been attributed to some 2S albumins, namely antifungal properties.

Jug r 1

Jug r 1 protein presents a primary sequence of 139 aa that is fully represented in Table 1. This protein has an estimated molecular weight of 16.4 kDa and it is encoded by the nucleotide sequence *Juglans regia* 2S albumin seed storage protein precursor mRNA (Table 1) with 649 bp [38, 47]. The native walnut Jug r 1 is composed by two polypeptide

Table 1 Identification of common walnut allergens according to their biological function, clinical relevance and respective accession numbers [47, 55]

Allergen	MW (kDa)	Isoallergens	Isoforms or variants	Biochemical classification	Biological Function	Clinical relevance	Nucleotide (NCBI) [47]	Protein (NCBI) [47]	Protein sequence (UniProt) [55]
Jug r 1	16.4 (139 aa)	Jug r 1.01	Jug r 1.0101	2S albumin (prolamin superfamily)	Storage of nutrients for plant growth	Major allergen. Severe and systemic allergic reactions	U66866.1	AAB41308.1	AALLVALLFVANAAAFRTTITTMEIDEDIDNPRRRGEGCREQIQRQQNLNHCQYYLRQQSRSGGYDEDNQRQHFRQCCQQLSQMDEQCQCEGLRQVVRRQQQQQGLRGEEMEEMVQSARDLPNECGISSQRCEIRRSWF (P93198)
Jug r 2	44 (593 aa)	Jug r 2.01	Jug r 2.0101	Vicilin (cupin superfamily)	Storage of nutrients for plant growth	Major allergen. Severe and systemic allergic reactions	AF066055.1	AAF18269.1	RGRDDDDEENPRDPREQYRQCQEYCRRQGQGQRQQQQCQIRCEERLEEDQRSQEERERRRGRDVDDQNPRDPEQRYEQCQQQCERQRRGQEQTLCRRRCEQRRQQEERERQRGRDRQDPQQQYHRCQRRCQIQEQSPERQRQCQQRCERQYKEQQGRERGPEASPRRESRGREEEQQRHNPYYFHSQSIRSRHESEEGEVKYLERFTERTELLRGIENYRVVILDANPNTSMLPHHKDAESVAVVTRGRATLTLVSQETRESFNLECGDVIRVPAGATVYVINQDSNERLEMVKLLQPVNNPGQFREYYAAGAKSPDQSYLRVFSNDILVAALNTPRDRLERFFDQQEQREGVIIRASQEKLRALSQHAMSAGQRPWGRRSSGGPISLKSESPSYSNQFGQFFEACPEEHRQLQEMDVLVNYAEIKRGAMMVPHYNSKATVVVYVVEGTGRYEMACPHVSSQSYEGQGRREQEEEESTGRFQKVTARLARGDIFVIPAGHPIAITASQNENLRLLGFDINGENNQRDFLAGQNNIINQLEREAKELSFNMPREEIEEIFESQMESYFVPTERQSRRGQGRDHPLASILDFAFF (Q9SEW4)
Jug r 3	11.8 (119 aa)	Jug r 3.01	Jug r 3.0101	Non-specific lipid transfer protein 1 (prolamin superfamily)	Transference of lipids across membranes. Antimicrobial activity.	Major allergen. Severe and systemic allergic reactions	EU780670.1	ACI47547.1	MTGSLVLKLSGMVLLCMVVAAPVAEAVITCGQVASSVGSCIGYLRGTVPTVPPSCCNGVKSLNKAAATTADRQAACECLKKTSGSIPGLNPGLAAGLPGKCGVSVPYKISTSTNCKAVK (C5H617)
Jug r 4	58 (507 aa)	Jug r 4.01	Jug r 4.0101	Legumin (cupin superfamily)	Storage of nutrients for plant growth	Major allergen. Severe and systemic allergic reactions	AY692446.1	AAW29810.1	MAKPILLSIYLFLIVALFNGCLAQSGGRQQQQFGQCQLNRLDALEPTNRIEAEAGVIESWDPNNQQFQCAGVAVVRRTIEPNGLLLPQYSNAPQLVYIARGRGITGVLFPGCPETFEESQRQSQQGQSREFQQDRHQKIRHFREGDIIAFPAGVAHWSYNDGSNPVVAISLLDTNNNANQLDQNPRNFYLAGNPDDEFRPQGQQEYEQHRRQQQRQQRPGEHGQQQRGLGNNVFSGFDADFLADAFNVDTETARRLQSENDHRRSIVRVEGRQLQVIRPRWSREEQEREERKEREREREESESERRQSRRGGRDDNGLEETICTLRLRENIGDPSRADIYTEEAGRISTVNSHTLPVLRWLQLSAERGALYSDALYVPHWNLNAHSVVYALRGRAEVQVVDNFGQTVFDDELREGQLLTIPQNFAVVKRARNEGFEWVSFKTNENAMVSPLAGRTSAIRALPEEVLATAFQIPREDARRLKFNRQESTLVRSRPSRSRSSRSERRAEV (Q2TPW5)
Jug r 5	No data	No data	No data	Profilin	Binds actin, affects the structure of cytoskeleton	No data	No data	No data	No data

chains of 3.5 kDa (light chain) and 8 kDa (heavy chain), respectively, coupled to two inter-chain disulphide bridges and non-covalent bonds, resulting from the processing of a single chain precursor of 14 kDa [48]. Besides the two inter-chain disulphide bridges, the protein has two intra-chain disulphide bonds in the heavy chain that contribute to its globular conformation. Similarly to other 2S albumins, the tri-dimensional structure attributed to the native Jug r 1 consists of four alpha-helices connected by loops to spiral regions [48]. Four epitopic regions have been identified and characterised in native Jug r 1, corresponding to stretches ^{6}IDNPRR^{11} (epitope #1, with 6 aa), ^{42}YDEDNQRQH^{50} and ^{72}QVVRRQQQQ^{80} (epitopes #2 and #3 respectively, with 9 aa each), and ^{102}CGISSQRCEIRR^{113} (epitope #4, with 12 aa). These epitopic regions have charged residues that coincide with the exposed electropositively (epitopes #1, #3 and #4) and electronegatively (epitope #2) charged areas on the surface of the allergenic protein. Other small IgE-binding epitopic stretches were identified during the epitope mapping of Jug r 1, namely ^{18}EQI^{20}, ^{24}QNL^{26}, ^{30}QYY^{32} and ^{54}CCQ^{56}, although residues ^{24}QNL^{26} and ^{30}QYY^{32} have little exposure on the surface of the protein, thus being considered of poor contribution to the allergenicity of this protein. The other two regions (^{18}EQI^{20} and ^{54}CCQ^{56}) are located near the epitopes #1 and #2, respectively, suggesting a more close participation of these residues in the IgE-binding of walnut allergic patients [48].

Teuber et al. [49] was the first to report the successful cloning of the recombinant Jug r 1 from common walnut into the frame-shifted plasmid expression vector using the Jug r 1 cDNA, with a purified fusion protein detected as a band of approximately 42 kDa. The recombinant Jug r 1 presented a deduced molecular weight of 15-16 kDa, considering that the synthesis of foreign proteins expressed by the plasmid expression vector as a fusion product with a 26 kDa glutathione-S-transferase. The 2S albumin seed storage protein precursor (recombinant Jug r 1) with 139 aa in length exhibited a significant homology with the allergenic methionine-rich 2S albumin seed storage precursor from Brazil nuts, revealing 46% of identity with Ber e 1 [49]. Epitope mapping of the recombinant Jug r 1 evidenced that a linear stretch of 12 aa (^{33}QGLRGEEMEEMV^{44}) of the large subunit was responsible for the strong IgE-reactivity observed with the sera of walnut allergic patients. Moreover, the presence of the core amino acids ^{36}RGEE^{39} and an additional residue of glutamic acid at position 42 are determinant for IgE-binding to occur [50].

More recently, Sordet et al. [48] described the production of a correctly folded and fully functional recombinant Jug r 1. The recombinant allergen consisted of an uncleaved single chain precursor, thus differing from the native protein that is composed by two covalently linked light and heavy chains resulting from the *in planta* processing of the single chain precursor. Although not submitted to processing, the recombinant Jug r 1 precursor evidenced an overall fold and a disulphide bonding profile that is virtually identical to the native protein. In accordance with these structural similarities, both native and recombinant proteins exhibited a very close IgE-binding reactivity suggesting that the recombinant Jug r 1 could be a useful tool for replacing native Jug r 1 in the component-resolved diagnosis of walnut allergy. Jug r 1 presented high sequence identity with other 2S albumins, namely with Ana o 3 from cashew nut (identity of 55% and similarity of 91%) [51] and with Car i 1 from pecan nut (identity of 88% and similarity of 92%) [52]. The high degree of sequence identity among 2S albumins from different plant species contributes to the immunological cross-reactivity between Jug r 1 and other allergenic 2S albumins. In this case, similar conformational and/or shared linear epitopes are pointed as strong reasons for the cross-reactivity phenomena observed among these proteins [53]. 2S Albumins present high pH and thermal stability (original folding at temperatures >90ºC), mostly attributed to their compact and globular conformational structure, which is probably responsible for the resilient allergenic activity related to them [53, 54]. Supporting these evidences, the native Jug r 1 proteins reveal elevated resistance to enzymatic activity (trypsin/chymotrypsin) at basic pH (8.0), although they progressively lose allergenicity at acidic pH (1.3) in the presence of pepsin. In addition, native Jug r 1 molecules preserve their alpha-helical fold after heating at 90ºC and subsequently being cooled at 20ºC, which states a good resistance to heat denaturation [48]. Attending to these facts, the poor proteolysis of Jug r 1 at basic pH could contribute to the allergenic activity attributed to this allergen as well as other 2S albumins.

Jug n 1

Jug n 1 is an allergen identified in black walnut, whose primary sequence is constituted by 161 aa, being fully represented in Table 2. Classified as a 2S albumin, this protein is encoded by a nucleotide sequence of 574 bp with an estimated molecular weight of 18.9 kDa [38, 55]. A BLAST search indicates that this allergen presents elevated sequence identity with other nuts, namely 96% with Jug r 1 (*Juglans regia*), 87% with Car i 1 (*Carya illinoinensis*) and 57% with Cor a 14 (*Corylus avellana*) [38, 47]. The shared homology of those allergens could partly explain the cross-reactivity observed among these different nuts. Although the information about this allergen is very scarce, Ling et al. [56] reported the cloning of a protein with 234 aa (Jug n 1) from a cDNA clone of black walnut library with 749 bp presenting IgE-reactivity with sera from walnut allergic patients. In addition, this cloned protein also evidenced 83%, 82% and 81% of sequence homology with rubber tree manganese superoxide dismutase (MnSOD), rice MnSOD and tobacco MnSOD, respectively. Still, more detailed information is needed for a more precise characterisation of Jug n 1 allergen of black walnut.

Table 2 Identification of black walnut allergens according to their biological function, clinical relevance and respective accession numbers [47, 55]

Allergen	MW (kDa)	Isoallergens	Isoforms or variants	Biochemical classification	Biological Function	Clinical relevance	Nucleotide (NCBI) [47]	Protein (NCBI) [47]	Protein sequence (UniProt) [55]
Jug n 1	(161 aa)	Jug n 1.01	Jug n 1.0101	2S albumin (prolamin superfamily)	Storage of nutrients for plant growth	Major allergen Severe and systemic allergic reactions	AY102930.1	AAM54365.1	RHEARKCIFHTFSLTMARLATLAALLVALLFVANAAAFRTTITTMEIDEDIDNPRRRGEGCQEQIQRQQNLNHCQYYLRQQSRSGGYDEDNQRQHFRQCCQQLSQIEEQCQCEGLRQAVRRQQQQQGLRGEEMEEMVQSARDLPKECGISSQRCEIRRSWF (Q7Y1C2)
Jug n 2	55.7 (481 aa)	Jug n 2.01	Jug n 2.0101	Vicilin (cupin superfamily)	Storage of nutrients for plant growth	Major allergen Severe and systemic allergic reactions	AY102931.1	AAM54366.1	GRDRQDPQQQYHRCQRRCQIQEQSPERQRQCQQRCERQYKEQQGRERGPEASPRRESKGREEEQQRHNPYYFHSQSIRSRHESEEGEVKYLERFAERTELLRGIENYRVVILDANPNTFMLPHHKDAESVIVVTRGRATLTLVSQETRESFNLECGDVIRVPAGATEYVINQDSNERLEMVKLLQPVNNPGQVREYYAAGAKSPDQSYLRVFSNDILVAALNTPRDRLERFFDQQEQREGVIIRASQEKLRALSQHAMSAGQRPWGRRSSGGPISLKSERPSYSNQFGQFFEACPEEHRQLQEMDVLVNYAEIKRGAMMVPHYNSKATVVVYVVEGTGRYEMACPHVSSQSFEDQGRREQEEEESTGRFQKVTARLARGDIFVIPAGHPIAITASQNENLRLLGFGINGENNQRNFLAGQNSIINQLEREAKELSFNMPREEIEEIFESQMESYFVPTERQSRRGQGRDHPLASILGFAFF (Q7Y1C1)

nsLTP family

As part of the prolamin superfamily, the nsLTP are classified as important plant seed allergens, although these proteins have also been identified in other plant tissues (fruits, leaves, roots and pollen) [43, 57]. The nsLTP are biochemically defined as monomeric proteins of low molecular size, with primary sequences rich in cysteine residues, contributing to secondary structures composed by alpha-helices that involve a lipid binding cavity in the core. Members of this family share common structural features encompassing a characteristic eight cysteine motif (four disulphide bonds) and basic isoelectric points (pI~9) [58, 59]. The nsLTP are divided into two subfamilies of 9 kDa (nsLTP 1) or 7 kDa proteins (nsLTP 2), both with biological functions related to the transport of different classes of lipids (fatty acids, phospholipids, glycolipids and sterols) through membranes [57, 59]. Although performing identical tasks, proteins from the two subfamilies present a low overall amino acid sequence similarity, sharing only approximately 30% of identity [59]. To the nsLTP, other biological functions can also be attributed, namely active roles in plant protection (antifungal and antibacterial properties) [60] or potential involvement in plant growth and development (embryogenesis, germination) [58, 61]. With a wide distribution throughout nature [57], the nsLTP are commonly stimulated by biotic and abiotic plant stress factors, which is a typical trait of the group of pathogenesis-related (PR) proteins. As consequence, nsLTP are also known as the PR-14 protein family [43].

The nsLTP are highly resistant to heat treatment and to proteolytic digestion, which enables these molecules to remain intact during food processing and to survive to the harsh environment of the gastrointestinal tract. These characteristics, together with their great dissemination among organisms, make nsLTP regarded as pan-allergens, being even proposed as model allergens for true food allergy [62-64].

Jug r 3

Included in the nsLTP, the Jug r 3 protein has been classified as a food allergen in walnut seeds [38, 39]. It presents a primary structure of 119 aa (Table 1) encoded by the nucleotide sequence *Juglans regia* nonspecific lipid transfer protein mRNA with 360 bp [47]. With an isoelectric point of 9.45 and a molecular size of 9.1 kDa, the purified LTP seems to exclude any glycosylation. The N-terminal sequence of the first 30 amino acids exhibited a high degree of homology (80%) with other fruits, namely peach and apricot [65]. According to the NCBI database [47], upon protein alignment of relevant allergenic nsLTP from different species, Jug r 3 exhibited a sequence identity of 60% with Cor a 8 from hazelnut (accession no. AAK28533.1), 59% with Pru av 3 from cherry (accession no.

AAF26449.1), 57% with Ara h 9 from peanut (accession no. ABX56711.1) and 53% with Pru du 3 from almond (accession no. ACN11576.1). Due to this apparent relationship between Jug r 3 and other allergens from fruits/seeds of the Rosaceae family (almond, cherry, peach), cross-reactivity among them is potentially high [66]. The close sequence homology of LTP allergens from Jug r 3 and peach was evidenced by the IgE cross-reactivity between these proteins, suggesting the IgE-binding epitopes of walnut LTP are also present in peach LTP [65]. The nsLTP are mainly located in the outer epidermal layers of fruits such as peach, which in some cases allow allergic patients to tolerate peel-off fruits. However, in the case of nuts that are often eaten without removing the outer layer (peel), the allergic patients are still at risk of developing adverse immunologic reactions upon their consumption [67]. The effect of food processing on Jug r 3 allergen is not yet clear, although heat treatments above 90ºC during long periods seem to reduce the allergenicity of some allergenic nsLTP such as Mal d 3 from apple [68] and Cor a 8 from hazelnut [69].

Cupin superfamily

This superfamily encompasses a large and multifunctional variety of proteins that are estimated to be originated by divergent evolution from a common ancestor, whose presence is transversal to different kingdoms (Bacteria, Plants and Animals) [46, 70]. These proteins were collectively designated as cupins due to the existence of a beta-barrel motif in their tri-dimensional structure [71]. Based on the presence of one or two cupin domain(s), the members of this superfamily are classified as mono- or dicupins, respectively. The functional class of dicupins includes the 7S and 11S globular seed storage proteins, which represent major protein components of several plant foods, namely tree nuts. Owing to their different sedimentation coefficients, globulins are divided in two groups: the vicilins (trimeric 7S globulins) and the legumins (hexameric 11S globulins) [40, 72].

Vicilin family (7S globulins)

The vicilins-like proteins belong to the cupin superfamily, presenting a structure of two conserved beta-barrel motifs classifying them as bicupins. Included in the vicilin family of proteins, the Jug r 2 and Jug n 2 were identified as allergens of *Juglans regia* and *Juglans nigra,* respectively. The 7S globulins are proteins with a total molecular weight of 150-190 kDa and a trimeric structure, being composed of three subunits with molecular weights ranging from 40 to 80 kDa each [73]. Vicilins lack residues of cysteine in their primary sequence [74], thus the structural stability of these proteins is ensured by non-covalent hydrophobic interactions, hydrogen bonds and van der Waals interactions. The 7S globulins are structurally composed by two N-terminal and C-terminal domains comprising a beta-barrel motif in each domain. Unlike the 11S globulins, the vicilins are commonly glycosylated, with one or two N-linked glycosylation sites located in the C-terminal domain [75]. In legumes, vicilins are considered important seed storage proteins, playing a role as nitrogen donor during seed germinating and acting as plant protection proteins (namely antifungal activity) [76].

Jug r 2

The Jug r 2 protein has been identified as an important allergen in common walnut [38, 39]. The primary structure of this protein presents 593 aa (Table 1) encoded by the nucleotide sequence *Juglans regia* vicilin-like protein precursor, mRNA with 2057 bp [47]. The recombinant protein Jug r 2 was first reported by Teuber et al. [76], who subcloned the Jug r 2 cDNA into the frame-shifted plasmid vector pGEX-4T-3. Considering that recombinant Jug r 2 is expressed in a fusion protein (92 kDa) containing glutathione-S-transferase of 26 kDa, the estimated molecular weight for the cloned Jug r 2 was 66 kDa. Since Jug r 2 cDNA encodes a proprotein, the molecular weight of the mature vicilin is about 47 kDa (44 kDa in 12% acrylamide) after the cleavage of the precursor at amino acid position 173 [76]. Jug r 2 is considered a complex trimeric protein like several other plant vicilins. Similarly to the 7S globulins of soybean (Gly m 5) and peanut (Ara h 1), Jug r 2 from walnut was also classified as an allergen. A BLAST search evidences a high sequence similarity of this protein, not only with the allergic vicilin from black walnut (Jug n 2), but also with different plant sources, namely other tree nuts. Accordingly, Jug r 2 exhibits a sequence identity of 97% with Jug n 2 (black walnut, accession no. AAM54366.1), 47% with Cor a 11 (hazelnut, accession no. AAL86739.1), 43% with Ses i 3 (sesame, accession no. AAK15089.1) and 38% with Pis v 3 (pistachio, accession no. ABO36677.1) [47]. Jug r 2 also presents 92% of sequence similarity with Car i 2 allergen (accession no. ABV49590.1), though this pecan nut vicilin was not yet included in the IUIS list of allergens [38, 47].

As consequence of close sequence similarity and identity of Jug r 2 with other vicilins, Barre et al. [77] reported the characterisation of surface-exposed IgE-binding epitopes on the molecular organisation of tri-dimensional models of the vicilin allergens of walnut (Jug r 2), hazelnut (Cor a 11), cashew nut (Ana o 1) and peanut (Ara h 1). When comparing the IgE-binding epitopes of those allergenic vicilins, Jug r 2 was found to be structurally more closely related to Ara h 1, presenting an overall sequence identity of 54% with the latest [77]. With similar conformational IgE-binding epitopes, cross-reactivity occurrences among vicilins from different plant sources are expected.

Vicilins exhibit considerable thermal stability, which allows maintaining their conformations at temperatures below 70-75ºC [75]. Still, when submitted to high temperatures, 7S globulins may suffer structural disruptions and covalent modifications with special emphasis for those involved in glycation processes or Maillard rearrangements [75]. Depending on the type of food processing, these alterations are likely to affect the allergenicity of vicilins, which can even be potentially increased. The allergenicity of Jug r 2 was evaluated after treating walnuts with single or combined cooking processes, which included gama-irradiation (1-25 kGy), microwave (500 W, 1 and 3 min), roasting (138ºC and 160ºC – 30 min; 168ºC and 177ºC – 12 min), frying (191ºC, 1 min), blanching (100ºC, 5 and 10 min) and/or autoclaving (121ºC, 15 psi, 15 and 30 min) [78]. With this study, it was verified that walnuts were antigenicity stable to all the tested procedures as indicated by the stability of the polypeptide profiles. Additionally, the results confirmed that roasting, frying above 191ºC or microwave heating had no significant effect on immunoreactivity of walnut. After autoclaving, the immunoreactivity of walnut proteins tested by ELISA was apparently not affected, though results of Western blot analysis showed a decrease on the recognition of 42-45 kDa Jug r 2 proteins.

Jug n 2

An important allergen in black walnut corresponds to the protein Jug n 2, which has been identified as a vicilin from the cupin superfamily. This protein, presenting an estimated molecular weight of 55.7 kDa, is encoded by the *Juglans nigra* vicilin seed storage protein mRNA with 1732 bp and it is composed by a primary structure of 481 aa, which is fully represented in Table 2 [38]. Like for Jug r 2, Jug n 2 also exhibits elevated sequence identity with other allergenic vicilins, namely 92% with Car i 2 (accession no ABV49590.1), 46% with Cor a 11 and with Ses i 3 (accession no AAL86739.1 and AAK15089.1, respectively) and 39% with Pis v 3 (accession no ABO36677.1) [47]. Regarding Jug n 2 allergen, little information has yet been made available. Ling et al. [56] reported the cloning of a protein with 338 aa (Jug n 2) from a sequence of cDNA of black walnut library with 1317 bp. The produced protein evidenced IgE-reactivity with sera from walnut allergic patients. In addition, the cloned protein also exhibited 84% and 83% of sequence homology with Pea and Tobacco glyceraldehydes, respectively [56]. Jug n 2 has been included in the IUIS list of allergens, which only occurs after careful evaluation of the allergen submission, according to the molecular and immunological requirements established for inclusion into the allergen nomenclature that is made by specialised members of the Executive Committee. However, in the case of Jug n 2 this information has not been made available for common researchers [38]. Therefore, further information about molecular characterisation and allergenicity assessment of Jug n 2 is still much needed.

Legumin family (11S globulins)

The other class of functional proteins included in the bicupins corresponds to the 11S globulins that are also designated as legumins. Mature 11S globulins are non-glycosylated hexameric proteins with a total molecular weight of approximately 360 kDa. The structure of legumins is determined by six monomers interacting non-covalently and displaced in an open ring conformation [40]. Each of the six monomers is initially synthesised as a unique polypeptide, being post-translationally cleaved into two polypeptide chains, one acidic with 30 to 40 kDa that is linked by a single intermolecular disulphide bond to a basic polypeptide of approximately 20 kDa [75]. The mature and functional 11S globulins (legumins) are rarely glycosylated, in opposition to other bicupins (e.g. vicilins).

Among tree nuts, since the edible part lies on the kernel, seed storage proteins are major components of the nutrient reservoir. As consequence, legumins and vicilins are considered important allergens in this group of seeds.

Jug r 4

Jug r 4 is a different member from the cupin superfamily classified as a 11S globulin (legumin-type). This allergen is encoded by a nucleotide sequence of *Juglans regia* seed storage protein mRNA with 1524 bp presenting a complete primary sequence composed by 507 aa that is fully represented in Table 1 [38]. The cloning of walnut cDNA into a maltose binding protein (MBP) expression vector pMAL-c2X and transformed into *Escherichia coli* was reported by Teuber et al. [79] and Wallowitz et al. [80]. With the first 23 aa residues predicted to be a signal peptide, the open reading frame was 507 aa in length, corresponding to a molecular weight of 58.1 kDa and an isoelectric point of 6.8. The estimated molecular size relates to a single subunit of walnut legumins since these proteins are hexamers (six subunits) [80]. Sequence identity among allergenic legumins from different plant sources is also frequently high. The results from multiple sequence alignments of Jug r 4 with other allergenic legumins revealed 95% identity with Car i 4 from pecan nut (accession no. ABW86978.1), 72% with Cor a 9 from hazelnut (accession no. AAL73404.1), 57% with Ana o 2 from cashew nut and Pis v 5 from pistachio (accession no. AAN76862.1 and ACB55490.1, respectively), 54% with prunin-2 that is part of Pru du 6 allergen from almond (accession no. ADN39441.1) and 51% with Ber e 2 from Brazil nut (accession no. AAO38859.1) [47]. From the comparison of these sequences it is possible to suggest that the majority of the differences between legumin proteins are predominantly located in one of three sections: the first 34 amino acids (including the signal peptide), amino acids 210-231, and the last residues, 500-518. According to Wallowitz et al. [80], the alignment of legumins from walnut, cashew, hazelnut and peanut suggests that the major cleavage site separating the protein into acidic (larger) and basic (smaller) subunits is predictably located between residues 315 and 316. The cleavage site containing an Asn-Gly peptide bond seems to be well conserved among a wide variety of plant species. Jug r 4 and several other legumins possess the NGXEET motif: NGLEET is characteristic in Jug r 4 from walnut and in Pru du 6 from almond, NGFEET in Cor a 9 from hazelnut, NGIEET in Ana o 2 from cashew and in Ara h 3 from peanut [81]. Linear IgE-binding epitopes identified in legumins from tree nut group and peanut have been mapped using tri-dimensional models of the Jug r 4 (walnut), Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Ara h 3 (peanut) proteins. The analysis of the conformational organisation of these legumins revealed some structural homology on the surface-exposed epitopes that could explain the IgE-binding cross-reactivity observed among tree nut allergens [82]. These evidences support the occurrence of cross-reactivity between the recombinant Jug r 4 allergen and protein extracts from hazelnut (Cor a 9), cashew (Ana o 2) and peanut (Ara h 3) [80]. More recently, Robotham et al. [83] sequenced the four epitopic regions (HS#1 to HS#4) most frequently recognised by patient IgE, terming these binding epitopes as "hot spots" and mapping the antigenic surfaces onto tri-dimensional models. HS#2 and HS#4 IgE-binding epitopes share primary sequence similarity between Jug r 4 and Cor a 9, (100% for HS#2 and 86% for HS#4) and appear to be structurally related as evidenced by similarities in the surface topology and charge distribution regions [83]. Epitopic region HS#1 is also similar among Jug r 4 and other legumins, namely Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Gly m 6 (soybean), but not with Ara h 3 (peanut). The majority of the residues are located in the inner part of the structure, indicating that this epitopic region is only available when the monomeric subunit is denatured and/or fragmented [83].

Like the vicilins, the legumins share a propensity to form large thermally induced aggregates with high tendency to mould heat-set gels and function as emulsifiers [54]. In general, legumins present, not only an elevated thermal stability responsible for preserving their conformational structures at temperatures below 94ºC, but also high resistance to proteolysis, which retaining their allergenic properties along the gastrointestinal system [75]. The general treatments used in food processing such as heat (roasting, blanching, and autoclaving) and radiation (gama and microwave) are not likely to alter the immunoreactivity of Jug r 4 protein. As demonstrated by Su et al. [78], only the combination of gama-irradiation (25 kGy) and autoclaving for 30 min show a reduction in the recognition of proteins of 45-66 kDa (identified as Jug r 4) by Western blot analysis, but not when tested for immunoreactivity by ELISA [84].

Profilins

Profilins assemble a family of small cytosolic molecules (12-15 kDa), presenting highly conserved proteins that share high sequence identities (>75%), even among organisms distantly related [57]. As consequence of the abundant sequence conservation, profilins exhibit highly similar structures and biologic functions [46]. Biochemically, profilin are actin-binding proteins involved in the dynamic turnover and restructuring of the actin cytoskeleton. They belong to the alpha-beta-class of proteins, evidencing a structure mainly composed by two alpha-helices and five-stranded anti-parallel beta-sheets. Profilins perform an important role in cell-motility through the regulation of actin microfilament polymerisation dynamics [46]. The restructuring of the actin cytoskeleton is essential for processes such as the organ development, the wound healing and the hunting down of infectious intruders by cells of the immune system. In plants, these proteins perform an active role in the cytokinesis, the cytoplasmatic streaming, the cell elongation and the growth of root hairs and pollen tubes [46, 57, 85]. In addition to actin, profilins have also been described to bind different ligands such as phosphoinositides and poly-L-proline stretches, which suggest their involvement in other biological processes, e.g. membrane trafficking and organisation, signalling pathways [57, 86]. Due to their wide participation in many essential cellular processes, these proteins can be virtually found in most cells of all eukaryotic organisms. Consequently, profilins are also considered as pan-allergens that are responsible for several of the observed cases of cross-reactivity between inhalant and food allergens [87].

In general, profilins are sensitive to heat denaturation and gastric digestion, suggesting that these allergens are usually associated with the consumption of raw or poorly processed foods (e.g. fruits) [88].

Jug r 5

Regarding Jug r 5 protein in walnut, very little information is available. This protein is classified as a food allergen by the ALLERGOME database [39], presenting IgE activity in the *in vitro* non-functional test and cross-reactivity with the allergenic profilin Lol p 12 from grass. It is present in walnut seed tissues, being its ingestion the common route of exposure [39]. Since Jug r 5 has not yet been included in the IUIS list of allergens [38], its classification as an allergen is rather imprecise.

CLINICAL RELEVANCE OF WALNUT ALLERGY

The most common symptoms related to IgE-mediated food allergies are urticaria, angioedema, pruritus, nausea and vomiting, abdominal pain or cramping, and diarrhoea. Respiratory symptoms such as sneezing, rhinorrhoea, nasal congestion, coughing, stridor, wheezing and ocular injection, often occur associated with cutaneous and gastrointestinal complaints [13]. The most severe IgE-mediated response to food is anaphylaxis that is a systemic and potentially fatal allergic reaction. Typically, this clinical presentation can occur suddenly upon the ingestion of the offending food allergen [89].

In spite of the fact that any food could virtually trigger a food allergy, eight groups foods are responsible for the most significant abnormal immunological responses, in which are included the groups of tree nuts and peanut [13]. Allergic reactions to peanut and tree nuts are frequently related to severe clinical presentations such as anaphylaxis, often resulting in fatalities [13, 90].

In the last years, significant research has been conducted in order to characterise the allergens involved in tree nut allergy. The categorisation of the specific allergens, both from a biochemical and immunological point of view, allowed them to be classified into families and superfamilies [45, 91]. In this context, pathogenesis-related proteins belonging to the Bet v 1-homologous family, lipid transfer proteins and structural profilins represent minor components in tree nuts, while the 2S albumins, legumins and vicilins are classified as important seed storage proteins in nuts. Allergens belonging to the seed storage proteins present high clinical relevance since they are often related to the most severe cases of tree nut allergic reactions such as anaphylaxis [91]. Walnut allergy is often severe and potentially life-threatening. Systemic reactions are frequent and commonly occur with complex clinical manifestations [91]. Among the tree nuts, walnuts, hazelnuts and almonds, are frequently cited as being the cause of systemic allergic reactions [92]. This fact is most likely linked to the presence of allergens belonging to the prolamin superfamily (2S albumins and nsLTP) and cupin superfamily (vicilins and legumins) [45, 91, 93].

In a study reported by Teuber et al. [49], the sera from 12 (out of 16) walnut allergic patients demonstrated IgE-binding to the 2S albumin seed storage precursor fusion protein (Jug r 1). This protein was suggested to be associated with severe symptoms because all of the 16 patients had a clear history of life-threatening systemic allergic reactions to walnuts. Considering that more than 50% of sera from walnut allergic patients were reactive to Jug r 1, this protein was defined as a major allergen in walnut [49, 94]. The high stability of their intrinsic protein structure enables the 2S albumins to cause sensitisation directly *via* the gastrointestinal tract. Moreover, stability to thermal processing was demonstrated for several 2S albumins [53]. Recent data indicated that 2S albumins are able to retain linear and potentially conformational epitopes following heat treatment up to 100°C. Consequentially, their ability to trigger an allergic reaction in sensitised individuals would essentially remain unaltered after thermal processing. Allergic reactions to 2S albumins from different plant sources such as Brazil nut, mustard, sesame and almond have been described with increasing frequency [53]. Although mild symptoms have been associated with this class of proteins, it is important to emphasise the relative frequency of the occurrence of severe reactions, including laryngeal angioedema and anaphylaxis. While 2S albumin storage proteins can be viewed as universal allergens among seeds, they are not necessary cross-reactive. Though high structural homology has been described, cross-reactivity seems to be uncommon in this family of proteins. In spite of this fact, cross-reactivity among 2S albumins from the Brassicaceae family, including oilseed rape, turnip rape and mustard has been demonstrated [53].

Jug r 2 is another seed storage protein that was classified as a major allergen in walnut [76]. This classification was endorsed to this protein since in the study, reported by Teuber et al. [76], 9 out of 15 sera of patients with severe and systemic walnut allergy were reactive to Jug r 2. Presenting high similarity (70%) with the homologue protein in peanut (Ara h 1), which is responsible for the majority of the reported cases of fatal anaphylaxis [45, 91], Jug r 2 is also expected to induced severe symptoms in walnut allergic patients. Despite the high similarity between Jug r 2 and Ara h 1, minimal *in vitro* cross-reactivity was demonstrated between peanut and walnut proteins. When testing patients with clear IgE reactivity to Jug r 2, no evidence of high-affinity cross-reactive IgE with Ara h 1 (crude peanut extract) could be observed [76].

Included in the nsLTP family, Jug r 3 has been officially recognised and identified as an allergen in common walnut [65]. Generally, the nsLTP are considered major cross-reactive allergens existing in the majority of the plant foods as well as in pollen of diverse plants. The route of sensitisation to these proteins is likely to be dependent on geographical differences. The clinical symptoms associated with nsLTP are normally classified as severe allergic reactions [57, 95]. In a study performed by Pastorello et al. [65] aiming at identifying the allergens involved in walnut allergy in Italian patients, 36 individuals out of a test population of 46 patients with mild (oral allergy syndrome) and severe clinical symptoms evidenced IgE reactivity to LTP (Jug r 3). Accordingly, the walnut allergic patients reacting to LTP often exhibited severe symptoms, being also allergic to other fruits containing LTP. Curiously in the referred test population, vicilins were considered as minor allergens, supporting the previous theories related to LTP routes of sensitisation. In this case, the sensitisation to this protein seems to be secondary to the sensitisation to peach LTP, which seems to act as the primary sensitizer to walnut allergy. The majority of the patients were sensitised to different plant foods other than walnut, but the patients who were exclusively allergic to walnut were sensitised to vicilins. These patients had several episodes of anaphylaxis and/or glottis oedema, even after ingestion of minute quantities of walnut, present as a hidden allergen. In the referred study, it was demonstrated that both LTP and vicilins are true food allergens because they were able to sensitise patients not allergic to pollen, triggering severe reactions [65].

The fourth allergen described for common walnut corresponds to Jug r 4, which is a legumin from the cupin superfamily. Teuber et al. [79] found that the sera of 15 individuals from a test population of 23 patients with severe and systemic reactions to walnut evidenced IgE reactivity to the fusion protein containing Jug r 4. This allergen proved to be a major allergen in walnut since more than 65% of the test population was reactant to it. Wallowitz et al. [80] used sera from 37 patients with histories of potentially life-threatening systemic reactions to walnut, involving bronchospasm, hypotension, or laryngoedema/throat swelling, which enabled verifying IgE reactivity with recombinant Jug r 4 in 21 individuals of the test population. In the referred study, more than 57% of the sera from walnut allergic patients were reactive to Jug r 4, thus confirming the previous designation of major allergen attributed to this protein. Additionally, Jug r 4 displayed significant sequence homology with other allergenic legumins, namely with Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Ara h 3 (peanut), contributing to cross-reactivity occurrences, as already was confirmed *in vitro* for those nuts.

For the black walnut species, two allergens have been officially recognised so far, a 2S albumin and a vicilin, respectively Jug n 1 and Jug n 2. Being genetically closely related, common and black walnut present elevated homology. With respect to this, Jug n 1 and Jug n 2 were found to be 96% and 97% identical to Jug r 1 and Jug r 2, respectively. Although black walnut is not usually consumed, Jug n 1 and Jug n 2 walnut allergens have been identified. Consequently, most walnut-allergic patients have probably been sensitised to the widely consumed common walnut rather than the black walnut [91]. In an interesting study performed by Comstock et al. [96], extensive *in vitro* cross-reactivity to seed storage proteins among different walnut cultivars and species was demonstrated. Consequently, patients with severe allergy to common walnut are expected to be clinically allergic to all commercial walnut cultivars and to other closely related species from *Juglans* genus.

The current research on the characterisation of allergenic components has opened new perspectives in the diagnosis of food allergy. Recently, relevant information on the biochemical classification of walnut allergens has become available. Moreover, research studies allowed the correlation between diverse groups of allergenic proteins in walnut and respective clinical symptoms elicited. However, more studies are still needed in order to better understand the different patterns of sensitisation. Though some patients seem to be exclusively sensitised to walnut, others report allergy to multiple nuts as well as additional foods or pollens. Whether such patterns of reactivity are the result of multiple independent sensitisations, which are frequent in atopic patients or derived from true cross-reactivity, it should be questioned and further investigated. From a clinical point of view, in the case of a patient sensitised to multiple foods, *in vivo* and *in vitro* tests should always be performed attempting to answer to this question. Additionally, the results of these tests are expected to be analysed in the context of geographic environment, dietary habits of the patients and cooking procedures. The role of food processing conditions, food matrices and the biochemical characteristics of the allergen itself should be investigated in terms of sensitisation and allergy. This research is fundamental for prescribing adequate food avoidance aiming at achieving a proper patient management.

With respect to the recent advances in recombinant DNA technology for allergen research, the application of recombinant allergens has been regarded as an excellent tool to improve allergy diagnosis procedures. With the categorisation of allergens in certain molecular families, it is now possible to predict the clinical relevance of the sensitisation. However, clinical studies on cross-reactivity between tree nuts are still scarce. For the moment, the avoidance of tree nuts besides walnut is highly recommended for sensitised individuals to walnut, unless specific challenges are performed to ensure clinical tolerance. Moreover, extra precaution should always be taken to avoid foods susceptible of containing hidden allergens as result of cross-contamination during processing [92]. In this context, it is critical that investigation based on collaborative studies between clinicians and researchers may continue, prompting to a better management of food allergies such as walnut allergy.

WALNUT DETECTION METHODS

Even when following a restrict diet with total avoidance of the offending foods, the sensitised/allergic individuals can still be at risk of suffering abnormal immune episodes as consequence of accidental exposure to hidden allergens in foods owing to incorrect labelling or cross-contaminations during food processing. Therefore, to verify labelling compliance [9, 10], to help the industrial management of food allergens and to ensure consumer’s safety, the development of proper and highly sensitive analytical methodologies has attained special emphasis [97].

Presently, the need for adequate methodology has prompted the development of numerous techniques for the evaluation of most allergenic ingredients. Nevertheless, the lack of available testing/reference materials and the absence of official methods for their detection and quantification represent main difficulties in the management of food allergens. In addition, the absence of consensus towards the best methodology for allergen detection is still a matter of extensive debate among researchers. Until now, protein- and DNA-based methods have been considered useful tools for the evaluation of allergenic ingredients in foods. Among the available techniques, the choice of a method lies on specific criteria such as target analyte (proteins versus DNA), basis of detection (e.g. chemical), cross-reactivity, setup cost, running cost, the need for expertise knowledge and possibility of multitarget detection [98]. Based on these criteria, protein-based methods as enzyme-linked immunosorbent assays (ELISA) or DNA-based techniques like polymerase chain reaction (PCR) are often preferred. Actually, a reasonable number of methods, either based on proteins or DNA, is available for the detection of walnut as an ingredient or a potential hidden component in foods.

Protein-based approaches

Within the protein-based methods, two main groups of techniques can be defined. One group encompassing the techniques based on allergen-antibody interactions such as lateral flow devises (LFD), ELISA, immunosensors and other relevant immunoassays (e.g. immunoblotting), and a second group covering the mass spectrometry (MS) platforms. So far, the methods based on the immunoassays are among the most popular, though recently the MS methods have also conquered a special role in the detection and quantification of allergens in foods.

Lateral Flow Devices or Dipsticks

This type of tests provides qualitative or semi-quantitative information that can be simply read visually. Besides this main advantage, the LFD are of simple and rapid performance, without needing specialised equipment or personnel [99]. Presently, three different LFD can be commercially acquired for the detection of walnut in foods (Table 3). The sensitivity of the LFD range from 2 to 10 mg/kg of walnut in foods, allowing the performance of the test in less than 15 min. Considering that for food industries the need for onsite quick results is very high, these strips are regularly used for allergen control, allowing to check the presence or absence of the offending food. However, the LFD present severe drawbacks such as the lack of providing quantitative information and the high propensity to false negative results, indicating that these tests should be confirmed with immunological methods of higher sensitivity and precision, namely the ELISA [100].

ELISA systems

As part of protein-based approaches, the immunochemical assays such ELISA are by far the most widely used, due to their direct assessment of the allergen and/or marker protein, low setup cost, moderated running time and no special requirements for expertise knowledge [98]. Additionally, the ELISA systems can provide quantitative information, which is one of the major requisites in allergen analysis. Resulting from these clear advantages, the immunoassays have been extensively applied to a wide variety of food allergens, in which walnut is included. Currently, some ELISA kits have been commercially available for the detection of walnut in foods (Table 3). They enable the detection of walnut protein down to 0.25-0.35 mg/kg in several food matrices such as cookies, ice-creams or chocolates (Table 3).

Table 3 Commercial ELISA, LFD and real-time PCR kits for the detection and quantification of walnut allergens

Commercial kits/Brand	Assay type	Cross-reactivity	LOD	LOQ	Estimated time to perform assay
Lateral Flow Walnut (R-Biopharm AG Darmstadt, Germany)	LFD	Pecan nut: 100 %	10 mg/kg	-	~15 min (sample preparation)
Walnut Protein Rapid Test (Elution Technologies, Vermont, USA)	LFD	No cross-reactivity observed	2 mg/kg	-	~10 min (applied to extracted sample)
AgraStrip® Walnut (Romer Labs Division Holding GmbH, Austria)	LFD	No available information about the specificity	-	-	~11 min
BioKits Walnut Assay Kit (NEOGEN Corporation, Michigan, USA)	Polyclonal antibody specifically detects walnut proteins, sandwich ELISA (48 wells)	Pecan (2.3%), Quinoa (0.0012%), Pistachio (0.0009%), Hazelnut (0.0005%), Buckwheat (0.00024%).	0.25 mg/kg	2.4–120 mg/kg	~40 min (sample extraction) + ~75 min (incubation time)
AgraQuant® Walnut Assay (Romer Labs Division Holding GmbH, Austria)	Quantitative - Sandwich ELISA	No available information about the specificity	0.35 mg/kg	2–60 mg/kg	~40 min (sample extraction) + ~60 min (incubation time)
Walnut Protein ELISA Kit (Elution Technologies, Vermont, USA)	Sandwich ELISA	No cross-reactivity observed	-	1 mg/kg	~60 min (applied to extracted sample)
Walnut ELISA Kit (Creative Diagnostics, Shirley, New York, USA)	Quantitative - Sandwich ELISA	8/35 species. Pecan nuts (0.8%), chestnut (0.11%), hazelnut (0.02%), soybean, pine nuts, pistachio, sesame and brazil nuts (0.0003%)	0.35 mg/kg	2 mg/kg	~60 min (applied to extracted sample)
Walnut, Food, BioAssay™ ELISA Kit (Juglans regia) (US Biological, Salem, Massachusetts, USA)	quantitative test ELISA - Sandwich ELISA	No available information about the specificity	0.35 mg/kg	-	~60 min (applied to extracted sample)
DAI Walnut ELISA (Diagnostic Automation /Cortez Diagnostics, Inc., Calabasas, California, USA)	Quantitative - Sandwich ELISA	8/35 species. Pecan nuts (0.8%), chestnut (0.11%), hazelnut (0.02%), soybean, pine nuts, pistachio, sesame and brazil nuts (0.0003%)	0.35 mg/kg	2 mg/kg	~60 min (applied to extracted sample)
SureFood Allergen Walnut (R-Biopharm AG Darmstadt, Germany)	Real-time PCR (qualitative)	Pecan nut	≤5 DNA copies, ≤0.4 mg/kg	10 DNA copies, 10 mg/kg	~35 min (applied to extracted sample)
NutsKit Real Time - Walnut and Pecan nut DNA detection Real Time PCR kit (InCura Srl, Casalmaggiore, Cremona, Italy)	Real-time PCR (qualitative)	0/20 plant- and animal-derived foods	1 copy of walnut or pecan nut	-	~35 min (applied to extracted sample)

However, in most kits the performance parameters state that the limit of detection (LOD) can vary according to the type of food matrix analysed. As expected, several ELISA kits present elevated cross-reactivity with other plant species, especially with pecan nut that belongs to the same botanic family (Juglandaceae) of walnut. Cross-reactivity with tree nuts (pistachio, hazelnut, Brazil nut, chestnut, pine nut) and other plant species (quinoa, sesame, buckwheat and soybean) are also referred to be frequent (Table 3).

Besides the commercial kits, non-competitive sandwich-type ELISA and indirect competitive ELISA have been proposed in the literature to trace walnut allergens or marker proteins in foods (Table 4). Doi et al. [101] developed an ELISA targeting the allergenic protein Jug r 1 by the use of rabbit antibodies raised against the 2S albumin protein fraction of raw walnuts from the Chandler cultivar. The assay presented high performance parameters with good recoveries for a wide variety of food matrices (e.g. bread, sponge cake, jelly and biscuit) spiked with 10 mg/kg of walnut. The proposed method was considered rapid, sensitive and specific for walnut detection, though strong cross-reactivity was observed in the presence of close related species such as pecan and hazelnut. Minor cross-reactivity was also observed for other nuts (Brazil nut, pistachio, macadamia, almond, cashew, pine nut) and plants (peanut and mustard).

The same ELISA approach was evaluated by Sakai et al. [102] in an interlaboratory study involving twelve different laboratories. Recovery, repeatability and reproducibility parameters were of good quality, reporting the suitability of the assay for walnut assessment. In spite of the high degree of food processing, recovery values were always in accordance with criteria of acceptance, emphasising the precision of the method [102]. Still, no improvement regarding cross-reactivity with pecan or hazelnut was apparently performed, which could restrict the potential application of the assay to processed foods susceptible of containing those nuts as hidden allergens. Niemann et al. [103] have also proposed an ELISA using rabbit and sheep polyclonal antibodies raised against raw and roasted common walnuts. The developed method enabled detecting down to 1 mg/kg of common walnut in several spiked samples, namely chocolates, cookies, muffins and ice-creams. Since the antibodies were raised against common walnut and not black walnut, the limit of quantification (LOQ) when using black walnut in the calibration curve was defined as 10 mg/kg. The specificity of the method was tested with 80 different foods and, as expected, some strong cross-reactivity was verified in the case of pecan nut. Mustard, hazelnut, poppy seeds and mace presented very small cross-reactivity, being considered almost negligible.

Table 4 Summary of the protein- based methods for the detection and quantification of walnut allergens in foods available in the literature.

Method	Antibody (immunisation)/target protein	Cross-reactivity	Sensitivity level	Applied food matrices	References
Sandwich ELISA	Rabbit antibodies (raised against walnut 2S albumin protein fraction).Walnut variety - Chandler	2/10 plant-derived foods. Strong cross-reactivity with pecan nut (100 mg/kg), hazelnut (1 mg/kg), brazil nut, almond, pine nut, peanut, cashew nut, pistachio, macadamia and mustard (<0.31 mg/kg which was the LOQ of the calibration curve)	LOQ<0.31 mg/kg and LOD<0.16 mg/kg of walnut protein defined by the calibration curve	Model processed foods containing 10 mg/kg of walnut (chicken meatballs, rice porridge, bread, sponge cake, orange juice, jelly, biscuit)	[101]
Sandwich ELISA	Rabbit antibodies (raised against walnut 2S albumin protein fraction).Walnut variety - Chandler (same system as Doi et al., 2008)	2/10 plant-derived foods. Strong cross-reactivity with pecan nut (100 mg/kg), hazelnut (1 mg/kg), brazil nut, almond, pine nut, peanut, cashew nut, pistachio, macadamia and mustard (<0.31 mg/kg which was the LOQ of the calibration curve)	LOQ<0.31 mg/kg and LOD<0.16 mg/kg of walnut protein defined by the calibration curve	Model processed foods containing 10 mg/kg of walnut (chicken meatballs, rice porridge, bread, sponge cake, orange juice, jelly, biscuit)	[102]
Sandwich ELISA	Polyclonal rabbit and sheep antibodies (raised against several varieties of raw and toasted English walnuts)	5/80 animal- and plant-derived foods and other ingredients. Cross-reactivity with pecan nut (40% of cross-reactivity with pecan for the same concentration), mustard, poppy seeds, hazelnut and mace (1 to 3 mg/kg)	1 mg/kg of walnut in several food matrices	Incurred standards (walnut in chocolate milk 0-100 mg/kg) Spiked samples (chocolates, muffins, cookies and ice-creams)	[103]
Indirect competitive ELISA	Rabbit polyclonal antibodies (raised against recombinant fusion protein Jug r 1).	0/22 plant- and animal-derived food. Considering that IC50 of walnut was 1.94 mg/kg and all the IC50 from other species were >1000mg/kg	LOD of 0.22mg/kg and LOQ of 0.44 mg/kg	Model mixtures of wheat, soybean, rice or corn powder spiked with walnut powder (10 mg/kg).	[104]
LC-ESI-LIT-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (2 Jur r 4 peptideS) (EFQQDR and LDALEPTNR)	Not verified	LOD=55 or 50 mg/kg and LOQ=180 or 160 mg/kg for LDALEPTNR (Jug r 4) peptide according to the MS^2 or MS^3 acquisition mode, respectively	Fortified samples of biscuits spiked with a mix of 5 nuts (peanut, hazelnut, walnut, almond and cashew). Cereal mixes, biscuits (commercial)	[109]
LC-ESI-LIT-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (2 Jur r 4 peptides) (ADIYTEEAGR and LDALEPTNR)	1 of the 2 Jug r 4 peptides (LDALEPTNR) in walnut also occurs in Car i 4 in pecan nut.	LOD=0.8 mg/kg and LOQ=2.6 mg/kg for ADIYTEEAGR (Jug r 4) in fortified biscuits. LOD=5 mg/kg and LOQ=18 mg/kg for LDALEPTNR (Jug r 4) in fortified dark chocolate.	Fortified samples of biscuits or dark chocolates spiked with a mix of 5 nuts (peanut, hazelnut, walnut, almond and cashew). Dark chocolates and biscuits (commercial)	[110]
LC-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (3 Jug r 1 peptides) (DLPNECGISSQR, QCCQQLSQMDEQCQCEGLR and GEEMEEMVQSAR)	Not verified	70 mg/kg of DLPNECGISSQR (Jug r 1) in bread material	Flour and bread spiked with allergen mix (almond, walnut, peanut, hazelnut, soybean, egg and milk).	[111]
LC-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (3 Jug r 1 peptides) (DLPNECGISSQR, QCCQQLSQMDEQCQCEGLR and GEEMEEMVQSAR)	Not verified	70 mg/kg of DLPNECGISSQR (Jug r 1) in bread material	Bread spiked with allergen mix (almond, walnut, peanut, hazelnut, soybean, egg and milk).	[112]

More recently, an indirect competitive ELISA was developed with polyclonal antibodies raised in rabbits against recombinant Jug r 1 allergen [104]. The method allowed detecting walnut Jug r 1 down to 0.22 mg/kg and quantifying the same protein down to 0.44 mg/kg in different matrices (rice, corn, soybean and common wheat). Cross-reactivity was performed with a total of 22 plant- and animal-derived foods, presenting high specificity for walnut.

The ELISA methods are totally dependent on the interaction between the antibodies and the specific proteins (allergens or marker proteins). On the basis of this interaction, strong cross-reactivity can occur among proteins from different sources, as it was already exposed in this section. Additionally, proteins are also prone to suffer conformational disruptions of their structures when submitted to harsh conditions such as those regularly used in food processing (e.g. heat treatments, glycation, pH alterations, formation of Maillard products, fermentation, partial hydrolysis). In this context, further research is still needed to evaluate the effect of different types of food processing on the capacity to detect walnut proteins as hidden allergens in foods.

MS platforms

The application of proteomic methodologies (allergenomics) focusing core technologies based on MS platforms has been lately exploited for the detection, quantification and characterisation of food allergens. The recent advances in modern MS equipment combined with effective bioinformatic tools are currently modernising the field of protein analysis [98]. MS-technology exhibits several advantages that highlight its potential application for allergen detection, such as the possibility of analysing target analytes with high sensitivity, accuracy, specificity and reproducibility [105]. Additionally, the application of MS platforms for allergen analysis over other technologies presents the benefit of allowing a large degree of freedom in the selection of a target analyte to determine the presence of the allergenic ingredient [106]. Methods based on MS-technology are less prone to problems related to cross-reactivity phenomena, which are very frequent in immunoassays, allowing the unequivocal confirmation of the identity of the tested proteins/peptides [107]. The relative and absolute quantification of proteins can be performed using one of two principles: analysis of intact proteins (analyte and reference standards) or analysis of peptides obtained from protein digestion using proteolytic enzymes [105]. Included in the latter principle, most of MS methods used in the identification of allergens and protein markers are carried out in “bottom-up” mode, being conducted on digested proteins by the use of proteases such as trypsin [107]. The quantification of the resulting peptides involves the separation of proteolytic fragments using reverse-phase liquid chromatography (LC) often coupled with electrospray ionisation tandem mass spectrometry (ESI-MS/MS) and multiple reaction monitoring (MRM) [106, 108]. Accordingly, the ability to target multiple allergens in single LC runs together with the high selectivity and rapid adaptability of MS methods highlight their adequacy for the analysis of allergens in foods. In spite of all well documented advantages, factors such as the elevated cost of the equipment and the requirement for specialised expertise to maintain MS platforms are likely to constrain the broad application of this technology [98].

Recently, some studies have been reported regarding the development of MS methods for the simultaneous detection of several allergenic foods, including walnut (Table 4). Using the LC coupled with electrospray ionisation (ESI) and linear ion trap (LIT) mass analyser, Bignardi et al. [109] advanced a method for the multiple detection of five nut allergens: Ana o 2 (cashew), Ara h 3/4 (peanut), Pru 1 (almond), Jug r 4 (walnut) and Cor a 9 (hazelnut) in foods. For the specific case of walnut, two proteolytic fragments (EFQQDR and LDALEPTNR) from Jug r 4 were targeted, but only the peptide LDALEPTNR was used for method validation. With this system, LOD of 55 mg/kg or 50 mg/kg and LOQ of 180 mg/kg or 160 mg/kg of Jug r 4 in spiked biscuits were achieved using MS^2 or MS^3 acquisition modes, respectively. The peptides used for the development of the MS method were carefully selected for unequivocal identification of the target allergen and compared with other peptide sequences using BLAST software. However, results from this search regarding possible cross-reactivity with tested protein sequences were not mentioned [109]. Based on the same MS method, the simultaneous detection of allergens from walnut, almond, hazelnut, cashew and peanut from biscuits and dark chocolates using a rapid size-exclusion solid-phase extraction step was described aiming at enhancing the sensitivity of the previous technique [110]. In this study, walnut peptides (ADIYTEEAGR and LDALEPTNR) were targeted in biscuits and dark chocolates respectively, under MS^2 acquisition mode. The selected method enabled a LOD and a LOQ of 0.8 mg/kg and 2.6 mg/kg, respectively, for peptide ADIYTEEAGR (Jug r 4) in biscuits. In dark chocolates, the target peptide was LDALEPTNR (Jug r 4), allowing its detection and quantification down to 5 mg/kg and 18 mg/kg, respectively. As expected, the sensitivities attained with dark chocolates were about one order of magnitude lower than in biscuits owing to increased complexity of the chocolate matrix. Cross-reactivity tests enable to confirm that walnut peptide (LDALEPTNR) also occurs in Car i 4 allergen from pecan nut [110], suggesting that the use of single peptides for unequivocal identification of the target proteins should be avoided. Targeting multiallergens, other LC-MS/MS method for the simultaneous detection of seven allergenic ingredients in foods was advanced [111, 112]. For the identification of walnut, three Jug r 1 peptides (DLPNECGISSQR, QCCQQLSQMDEQCQCEGLR and GEEMEEMVQSAR) were targeted in spiked matrices of bread and flour. The method allowed tracing 70 mg/kg of DLPNECGISSQR peptide (Jug r 1), both in incurred bread and flour, demonstrating elevated sensitivity of the proposed method for raw and processed food samples. However, when results from raw flour and processed bread were compared, the intensity of the signal for walnut, egg, soy and peanut decreased between 70 to 80%. In those cases, heat processing could have led to chemical modifications of the allergen, resulting in a mass shift that was not detected by the method. Additional influence of the matrix on the tryptic digestion could also explain the partial decrease of the signal verified in those allergens [111]. From these reports, it is important to refer that the application of MS technology to multiple allergen detection and quantification has high potential, though further research is still needed.

DNA-based

Although the methods based on the detection of the DNA do not target the allergenic proteins, they are able to target the respective encoding sequences or species-specific markers. The high stability of DNA upon pH alterations, partial hydrolysis or thermal treatment, which are processes commonly used by food industry, have elected this molecule as an ideal target for allergen analysis. In addition, the DNA-based techniques can be easily implemented in routine analysis and considered, at the same time, as confirmatory tools for the identification of allergenic foods [22, 113]. Therefore, a great number of detection and quantification DNA-based methods has emerged in recent years, demonstrating their potentialities and revolutionising the field of allergen analysis.

PCR systems

From the DNA-based methods, the ones relying on polymerase chain reaction (PCR) are among the most commonly used, due to their adequate setup cost, reasonable running time and moderate requirements for specialised equipment and personnel [98]. In addition to this, PCR methods are less prone to cross-reactivity phenomena since the chosen target sequence can be adapted, being independent on biological effects such as those occurring during antibody production.

Several PCR assays have been reported in the literature for the specific detection of walnut in processed foods, which are reported in Table 5. In opposition to numerous ELISA and LFD kits, only two commercially available real-time PCR systems could be found for the qualitative analysis of walnut in foods (Table 3).

Table 5 Summary of the reported DNA-based methods for the detection and quantification of walnut in foods.

Method	Target gene (NCBI accession number)	Fragment size (bp)	Cross-reactivity	Reference standards or model mixtures (range)	Sensitivity level	Applied food matrices	References
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	91	1/14 plant-derived foods. Cross-reactivity with pecan nut at Ct>35 cycles	Wheat powder spiked with walnut flour (100,000-10 mg/kg) Genomic walnut DNA (12,500-1.25 pg)	10 mg/kg 1.25 pg	No data	[114]
Hexaplex real-time PCR with hydrolysis probe	Jug r 2 (AF066055)	88	1/44 plant- and animal-derived foods. Cross-reactivity with carrot (0.18%)	Boiled sausages spiked with walnut (10,000-32 mg/kg) Rice cookies spiked with walnut (400-0 mg/kg)	100 mg/kg of walnut in boiled sausages 5 mg/kg of walnut in rice cookies	Spreads, chocolates, sauces, parfaits, pasta, sandwiches, lasagne	[115]
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	88	0/23 plant-derived foods	Cookies spiked with walnut (100,000-10 mg/kg)	100 mg/kg of walnut in cookies	Biscuits, wafers, cakes	[116]
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	88	0/23 plant-derived foods (tested by Brezna et al., 2006)	Oat flakes spike with nut mix (hazelnut and walnut) (100%-0%)	10%	Cakes	[117]
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	88	0/23 plant-derived foods (tested by Brezna et al., 2006)	Cake filling spiked with walnut (100%-0%)	10%	Nut cakes	[118]
Conventional PCR	matK (AF118027)	120	1/12 plant- and animal-derived foods. Cross-reactivity with pecan nut	No data	No data	Snacks, cookies	[119]
Ligation-dependent probe amplification (multitarget)	Jug r 2 (AF066055)	120	0/48 plant- and animal-derived foods	Walnut cookies were used as matrix	No data	Chocolates, yogurts, cookies, spreads, sausages	[120]
Single-tube nested real-time PCR (real-time PCR coupled with nested PCR)	Jug r 3 (EU780670)	136 99	0/31 plant-derived foods	Batter or sponge cakes spiked with walnut (500,000-1 mg/kg) Batter or sponge cake with 50% walnut serially diluted (100,000-1 pg)	10 mg/kg of walnut in batter or sponge cakes (LOD=LOQ) 1 pg of walnut in batter or sponge cakes (LOD=LOQ)	Sponge cakes	[121]
Optical thin-films biochips (multitarget)	Jug r 2 (AF066055)	91	No data	No data	Sensitivity was assessed with sesame but not with walnut	Bread sticks, wafers, biscuits, cookies, noodles	[124]

In the case of the SureFood real-time PCR kit, the stated absolute LOD is less than 5 DNA copies and the relative LOD is 0.4 mg/kg of walnut in several food matrices. However, as no calibration curve is used in this kit, it is not possible to confirm the alleged sensitivity. Moreover, the system presents cross-reactivity with pecan nut (Table 3). Regarding the NutsKit real-time PCR system, it was developed to target both walnut and pecan nut, presenting no cross-reactivity with other 20 foods. In relation to sensitivities, the available information regards the absolute LOD of the method, which corresponds to 1 DNA copy of walnut and pecan nut. The limitation of the NutsKit concerns its application to foods for discrimination purposes, which should be clearly avoided (Table 3).

One of the main advantages attributed to PCR methods regards their specificity. This is well evidenced in Table 5, in which the reported methods allow very small or no cross-reactivity to other non-target food species. Cross-reactivity only occurred at small extent in the case of pecan nut [114] or carrot [115]. Most of the presented real-time PCR methods were based on the amplification of a region encoding the allergenic protein Jug r 2 with 88 bp, which is considered rather adequate for real-time PCR using a hydrolysis probe to enhance specificity [115-118]. Relative sensitivities ranged from 10,000 mg/kg (10%) of walnut filling [117, 118] down to 5 mg/kg of walnut in rice cookies [115]. Using a conventional PCR approach, Yano et al. [119] targeted the matK gene, being able to detect and discriminate walnut from pecan nut by means of applying an additional step using a restriction enzyme (BfaI). Thus, walnut PCR amplicons were digested in two fragments that could be easily identified in agarose gel, while PCR products of pecan nut were not digested by BfaI. Although the technique can be performed for positive/negative detection of walnut, no attempt for establishing a sensitivity level was described in this study, which represents a major drawback for its application to commercial foods.

The development of multitarget PCR-based methods has also been focused in some studies. Köppel et al. [115] advanced a hexaplex real-time PCR system for the simultaneous detection of several allergens including walnut. The approach enabled detecting walnut down to 100 mg/kg in boiled sausages and 5 mg/kg in rice cookies, which is considered rather good sensitivity for allergen analysis. Another multitarget system based on ligation-dependent probe amplification (LPA) was proposed by Ehlert et al. [120]. Although declaring the quantitative detection of several allergens such as peanut, cashew, hazelnut and macadamia, no information was provided regarding sensitivity.

More recently, using a novel single-tube nested real-time PCR system that is based on the combination of real-time PCR and nested PCR approaches performed in a single tube, Costa et al. [121] developed a quantitative method for walnut determination on raw and processed material, namely batter and sponge cakes, respectively. The system targeted a nucleotide sequence encoding the Jug r 3 allergen that did not evidence any cross-reactivity with other tested food species. The application of the proposed system allowed a relative LOD of 10 mg/kg of walnut in batter and sponge cakes and an absolute LOD of 1 pg of walnut DNA (~1.6 DNA copies).

Genosensors and microarrays

The potential application of biosensors for the detection of allergens in foods has been regarded as an excellent alternative to the above described protein- and DNA-based techniques [122]. Biosensors are expected to solve several issues concerning simple, fast, reproducible and low cost multitarget detection, as well as answering to additional topics such as high speed of execution, easy to use and potential for automation. Therefore, it is predictable that in near future biosensors could be used at industrial scale for direct and real-time monitoring of allergens along a production line [122]. DNA sensors (genosensors) and DNA chips (microarrays) have emerged as valuable, easy, inexpensive, fast and selective tools with a wide application in very different fields [123]. While most of the proposed biosensors (often called immunosensors) are generally based on the recognition of the interaction antibody-allergen, the genosensors rely on the hybridisation of an immobilised DNA probe onto the transducer surface with the target DNA. When the probe hybridises with the target DNA, a signal is generated being subsequently measured. There are different types of transducers (optical, acoustical, amperometrical or potentiometrical) that are capable of acquiring the signal and further process it to give a proportional output to the concentration of a specific target. One of the main advantages attributed to this technology corresponds to its massive potential for the simultaneous detection of several targets, e.g. allergens in a multitarget basis. Moreover, reduction of reagents, costs and time of analysis, as well as the decrease of possible cross-contaminations, constitute additional benefits commonly indorsed to biosensors (genosensors).

In spite of the high potential for multitarget analysis, very few studies have been performed aiming at developing microarrays and DNA chips for the simultaneous detection of several allergens in a single assay. The study of Wang et al. [124] presented a silicon-based optical thin-film biochip for the simultaneous detection of eight food allergens, including walnut, on the basis of two tetraplex PCR assays. In this research, PCR fragments of 91 bp from Jur r 2 encoding gene were targeted to detect walnut, but without reporting respective sensitivity. In the same work, quantification of sesame with a LOD of 10 mg/kg was achieved, which suggests that the system should also be further investigated for the detection of other allergens such as walnut. The limited number of genosensors and microarrays applications for allergen analysis, indicates that much effort is still needed for its full development and virtual application on routine analysis, namely at industrial scale.

IMMUNOTHERAPY-INDUCED TOLERANCE

Food allergy develops as a result of failure or loss of oral tolerance, though naturally acquired tolerance to some allergenic foods may occur mainly in children [125, 126]. Allergies to milk and eggs are quite common among children of small ages, who mostly will outgrow their food allergy during childhood. Conversely, in the case of children suffering from allergies to peanut and/or tree nuts, they are more likely to be persistently affected by those allergies throughout adulthood [125]. The true mechanisms involved in naturally acquired tolerance are not yet fully understood, although recent data seem to suggest that in children with persistent egg or milk allergy, the IgE reactivity to linear epitopes is more relevant than with conformational ones [125]. Knowledge about the mechanisms of natural outgrowth of food allergy would certainly provide better understanding regarding the immune pathways that could be therapeutically targeted in patients with long-life persisting food allergy.

Therefore, pursuing definitive treatments for food allergy has been centred on strategies able to induce oral tolerance, being the oral immunotherapy (OIT) one of most promising approaches. At this time, the OIT is at preliminary stage, although few clinical trials have already been performed with tolerance as an outcome, mostly targeting eggs or milk allergies (see review [126]). It is also important to refer that clinical efficacy of OIT has also been demonstrated in studies using peanut extracts [127, 128]. Based on these evidences, OIT seems to present the potential to be applied for different allergenic foods, such as the case of walnut. Still, more extensive and appropriate research is required to provide potential definitive therapies for food allergic patients [126].

FINAL REMARKS

Walnut, along with other tree nuts, has been considered a very relevant allergenic food, affecting a small, but rather significant portion of the general population, especially in western countries. In general, clinical manifestations of walnut allergy are frequently severe and systemic potentially life-threatening, leading to anaphylaxis. The most widely consumed walnut species is *Juglans regia*, from which five groups of allergens have been identified and characterised, namely Jug r 1, Jug r 2, Jug r 3, Jug r 4 and Jug r 5. *Juglans nigra*, known as black walnut, is much less consumed than the common walnut, having two allergenic proteins identified, Jug n 1 and Jug n 2. With the exception of Jug r 5 that is only classified as food allergen by ALLERGOME database, all the other allergenic proteins from both walnut species were included in WHO/IUIS of allergens.

So far, the only effective means of preventing allergic reactions in sensitised individuals still continues to depend on the total avoidance of the offending food. In relation to this, the allergic individuals are highly reliant on proper food labelling when carefully choosing the processed foods that are commercially available. In this context, the control of labelling compliance is much needed, prompting the development of proficient analytical methodology (both protein- and DNA-based techniques) for allergen detection. Until now, no cure is yet available for food allergic individuals, although some clinical trials, namely oral immunotherapy, have been advanced in the past years aiming at defining possible therapeutics for the treatment of food allergies. This kind of studies is still at a preliminary stage and in a near future it is expected that forthcoming studies would include a range of important allergenic foods such as walnut and other relevant nuts.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2013. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

1 USDA, United States Department of Agriculture - Germplasm Resources Information Network, Beltsville, USA. Available at: http://www.ars-grin.gov/ Last accession on September 2013.

2 FAO, Food and Agriculture Organization of the United Nations, Rome, Italy. Available at: http://www.fao.org/. Last accession on September 2013.

3 Ros E. Health benefits of nut consumption. *Nutrients* 2010; **2**:652-82.

4 Inbaraj JJ, Chignell CF. Cytotoxic action of juglone and plumbagin: a mechanistic study using HaCaT keratinocytes. *Chem Res Toxicol* 2004; **17**:55-62.

5 FAOSTAT, The Statistics Division of the Food and Agriculture Organization of the United Nations, Available at: http://www.faostat.fao.org/. Last accession on September 2013.

6 Alasalvar C, Shahidi F. Tree nuts: composition, phytochemicals, and health effects: an overview. In: Alasalvar C, Shahidi F, eds. *Tree nuts: composition, phytochemicals, and health effects*. Boca Raton: CRC Press, 2008: 1-6.

7 CODEX STAN 1-1985. Amended in 1991, 1999, 2001, 2003, 2005, 2008 and 2010 regarding the general standard for the labelling of pre-packaged foods. FAO/WHO Standards, Off Codex Stand 2010; Rome, Italy. Available at: http://www.codexalimentarius.org/standards/list-of-standards/

8 The European Parliament and the Council of the European Union, Directive 2000/13/EC of 20 March 2000 relating to the labelling, presentation and advertising of foodstuffs. *Off J Eur Comm*; **L109**:29-42.

9 Commission of the European Communities, Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC regarding certain food ingredients. *Off J Eur Union*; **L310**:11-4.

10 The European Parliament and the Council of the European Union, Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. *Off J Eur Union*; **L304**:18-63.

11 Boyce JA, Assa'ad A, Burks AW *et al*. Guidelines for the Diagnosis and Management of Food Allergy in the United States: Report of the NIAID-Sponsored Expert Panel. *J Allergy Clin Immunol* 2010; **126**:S1-58.

12 Sicherer SH. Food Allergy. Mt Sinai J Med 2011; **78**:683-96.

13 Burks AW, Tang M, Sicherer S *et al*. ICON: Food allergy. *J Allergy Clin Immunol* 2012; **129**:906-20.

14 Taylor SL, Hefle SL. Food allergies and other food sensitivities, *Food Technol* 2001; **55**:68-83.

15 van Hengel AJ. Introduction. In: Nollet LML, van Hengel AJ, eds. *Food allergens: analysis instrumentation and methods*, Boca Raton: CRC Press, Taylor & Francis Group, 2011:1-11.

16 Sicherer SH Epidemiology of food allergy. *J Allergy Clin Immunol* 2011, 127:594-602.

17 Boye JI. Food allergies in developing and emerging economies: need for comprehensive data on prevalence rates. *Clin Trans Allergy* 2012; **2**:25.

18 Sicherer SH, Sampson HA. Food allergy: recent advances in pathophysiology and treatment. *Annu Rev Med* 2009; **60**:261-77.

19 Sicherer SH, Sampson HA. Food allergy. *J Allergy Clin Immunol* 2010; 125:S116-25.

20 Chafen JJS, Newberry SJ, Riedl MA *et al.* Diagnosing and managing common food allergies: a systematic review. *JAMA* 2010; **303**:1848-56.

21 Zuidmeer L, Goldhahn K, Rona RJ *et al*. The prevalence of plant food allergies: A systematic review. *J Allergy Clin Immunol* 2008; **121**:1210-8.e1214.

22 Costa J, Mafra I, Carrapatoso I, Oliveira MBPP. Hazelnut allergens: molecular characterisation, detection and clinical relevance. *Crit Rev Food Sci Nutr* (Accepted).

23 Gupta RS, Springston EE, Warrier MR *et al*. The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* 2011; **128**:e9-17.

24 Sicherer SH, Furlong TJ, Muñoz-Furlong A, Burks AW, Sampson HA. A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. *J Allergy Clin Immunol* 2001; **108**:128-32.

25 Sicherer SH, Muñoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol* 2003; **112**:1203-7.

26 Sicherer SH, Muñoz-Furlong A, Godbold JH, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol* 2010; 125:1322-6.

27 Soller L, Ben-Shoshan M, Harrington DW *et al*. Overall prevalence of self-reported food allergy in Canada. *J Allergy Clin Immunol* 2012; **130**:986-8.

28 Ortolani C, Ballmer-Weber BK, Hansen KS *et al*. Hazelnut allergy: A double-blind, placebo-controlled food challenge multicenter study. *J Allergy Clin Immunol* 2000; **105**:577-81.

29 Mills ENC, Mackie AR, Burney P *et al*. The prevalence, cost and basis of food allergy across Europe. *Allergy* 2007; **62**:717-22.

30 Burney P, Summers C, Chinn S, Hooper R, Van Ree R, Lidholm J. Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy* 2010; **65**:1182-8.

31 Madsen CB, Hattersley S, Allen KJ *et al*. Can we define a tolerable level of risk in food allergy? Report from a EuroPrevall/UK Food Standards Agency workshop. *Clin Exp Allergy* 2012; **42**:30-7.

32 Crevel RWR, Ballmer-Weber BK, Holzhauser T *et al*. Thresholds for food allergens and their value to different stakeholders. *Allergy* 2008; **63**:597-609.

33 Ward R, Crevel R, Bell I, Khandke N, Ramsay C, Paine S. A vision for allergen management best practice in the food industry. *Trends Food Sci Technol* 2010; **21**:619-25.

34 Taylor SL, Crevel RWR, Sheffield D, Kabourek J, Baumert J. Threshold dose for peanut: Risk characterization based upon published results from challenges of peanut-allergic individuals. *Food Chem Toxicol* 2009; **47**:1198-204.

35 Crevel RWR, Briggs D, Hefle SL, Knulst AC, Taylor SL. Hazard characterisation in food allergen risk assessment: the application of statistical approaches and the use of clinical data. *Food Chem Toxicol* 2007; **45**:691-701.

36 Blom WM, Vlieg-Boerstra BJ, Kruizinga AG, van der Heide S, Houben GF, Dubois AEJ. Threshold dose distributions for 5 major allergenic foods in children. *J Allergy Clin Immunol* 2013; **131**:172-9.

37 Eller E, Hansen TK, Bindslev-Jensen C. Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. *Ann Allergy Asthma Immunol* 2012; **108**:332-6.

38 ALLERGEN, official site for the systematic allergen nomenclature. Available at: http://www.allergen.org/. Accessed on March 2013.

39 ALLERGOME, allergome database, the platform for allergen knowledge, Latina, Italy. Available at: http://www.allergome.org/. Accessed on March 2013.

40 Breiteneder H. Classifying food allergens. In: Koppelman SJ, Hefle SL, eds. *Detecting allergens in food*. Boca Raton: CRC Press, 2006.

41 Mills ENC, Jenkins JA, Alcocer MJC, Shewry PR. Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Crit Rev Food Sci Nutr* 2004; **44**:379-407.

42 Kumar S, Verma AK, Das M, Dwivedi PD. Allergenic diversity among plant and animal food proteins. *Food Rev Int* 2012; **28**:277-98.

43 Egger M, Hauser M, Mari A, Ferreira F, Gadermaier G. The role of lipid transfer proteins in allergic diseases. *Curr Allergy Asthma Rep* 2010; **10**:326-35.

44 Pantoja-Uceda D, Bruix M, Santoro J, Rico M, Monsalve R, Villaba M. Solution structure of allergenic 2S albumins. *Biochem Soc Trans* 2002; **30**:919-24.

45 Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. *J Allergy Clin Immunol* 2000; **106**:27-36.

46 Hauser M, Egger M, Wallner M, Wopfner N, Schmidt G, Ferreira F. Molecular properties of plant food allergens: a current classification into protein families. *Open Immunol J* 2008; **1**:1-12.

47 NCBI, National Center for Biotechnology Information, Bethesda, USA. Available at: http://www.ncbi.nlm.nih.gov/ Last accession on September 2013.

48 Sordet C, Culerrier R, Granier C *et al*. Expression of Jug r 1, the 2S albumin allergen from walnut (*Juglans regia*), as a correctly folded and functional recombinant protein. *Peptides* 2009; **30**:1213-21.

49 Teuber SS, Dandekar AM, Peterson WR, Sellers CL. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* 1998; **101**:807-14.

50 Robotham JM, Teuber SS, Sathe SK, Roux KH. Linear IgE epitope mapping of the English walnut (*Juglans regia*) major food allergen, Jug r 1. *J Allergy Clin Immunol* 2002; **109**:143-9.

51 Teuber SS, Sathe SK, Peterson WR, Roux, KH. Characterization of the soluble allergenic proteins of cashew nut (*Anacardium occidentale* L.). *J Agric Food Chem* 2002; **50**:6543-9.

52 Sharma GM, Irsigler A, Dhanarajan P *et al*. Cloning and characterization of 2S albumin, Car i 1, a major allergen in pecan. *J Agric Food Chem* 2011; **59**:4130-9.

53 Moreno FJ, Clemente A. 2S albumin storage proteins: what makes them food allergens? *Open Biochem J* 2008; **2**:16-28.

54 Mills ENC, Sancho AI, Moreno J, Kostyra H. The effects of food processing on allergens. In: Mills C, Wichers H, Hoffmann-Sommergruber K, eds. *Managing allergens in food*. Boca Raton: CRC Press, 2007:117-33.

55 UniProt, Protein knowledgebase, UniProt Consortium. Available at: http://www.uniprot.org/ Last accession September 2013.

56 Ling M, Ye J, Beyer K *et al*. Cloning, identification, and epitope-mapping two black walnut (*Juglans niger*) allergens. *J Allergy Clin Immunol* 2003; **111**:S248.

57 Hauser M, Roulias A, Ferreira F, Egger M. Panallergens and their impact on the allergic patient. *Allergy Asthma Clin Immunol* 2010; **6**:1-14.

58 Kader JC. Lipid transfer proteins in plants. *Annu Rev Plant Physiol Plant Mol Biol* 1996; **47**:627-54.

59 Yeats TH, Rose JKC. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Sci* 2008; **17**:191-8.

60 Ebner C, Hoffmann-Sommergruber K, Breiteneder H. Plant food allergens homologous to pathogenesis-related proteins. *Allergy* 2001; **56**(S67):43-4.

61 Salcedo G, Sanchez-Monge R, Barber D, Diaz-Perales A. Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochim Biophys Acta* 2007; **1771**:781-91.

62 Asero R, Mistrello G, Roncarolo D *et al*. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2001; **124**:67-9.

63 van Ree R. Clinical importance of nonspecific lipid transfer proteins as food allergens. *Biochem Soc Trans* 2002; **30**:910-3.

64 Zuidmeer L, van Ree R. Lipid transfer protein allergy: primary food allergy or pollen/food syndrome in some cases. *Curr Opin Allergy Clin Immunol* 2007; **7**:269-73.

65 Pastorello EA, Farioli L, Pravettoni V *et al*. Lipid transfer protein and vicilin are important walnut allergens in patients not allergic to pollen. *J Allergy Clin Immunol* 2004; **114**:908-14.

66 Schocker F, Lüttkopf, D, Scheurer S *et al*. Recombinant lipid transfer protein Cor a 8 from hazelnut: A new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J Allergy Clin Immunol* 2004; **113**:141-7.

67 Fernández-Rivas M, Cuevas M. Peels of Rosaceae fruits have a higher allergenicity than pulps. *Clin Exp Allergy* 1999; **29**:1239-47.

68 Sancho AI, Rigby NM, Zuidmeer L *et al*. The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* 2005, **60**:1262-8.

69 López E, Cuadrado C, Burbano C, Jiménez MA, Rodríguez J, Crespo JF. Effects of autoclaving and high pressure on allergenicity of hazelnut proteins. *J Clin Bioinformatics* 2012, **2**:12.

70 Dunwell JM, Purvis A, Khuri S. Cupins: the most functionally diverse protein superfamily? *Phytochemistry* 2004; **65**:7-17.

71 Dunwell JM. Cupins: a new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnol Genet Eng Rev* 1998; **15**:1-32.

72 Radauer C, Breiteneder H. Evolutionary biology of plant food allergens. *J Allergy Clin Immunol* 2007; **120**:518-25.

73 Breiteneder H, Radauer C. A classification of plant food allergens. *J Allergy Clin Immunol* 2004; **113**:821-30.

74 Shewry PR, Napier JA, Tatham AS. Seed storage proteins: structures and biosynthesis. *Plant Cell* 1995; **7**:945-56.

75 Mills ENC, Jenkins J, Marigheto N, Belton PS, Gunning AP, Morris VJ. Allergens of the cupin superfamily. *Biochem Soc Trans* 2002; **30**:925-9.

76 Teuber SS, Jarvis KC, Dandekar AM, Peterson WR, Ansari AA. Identification and cloning of a complementary DNA encoding a vicilin-like proprotein, Jug r 2, from English walnut kernel (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* 1999; **104**:1311-20.

77 Barre A, Sordet C, Culerrier R, Rancé F, Didier A, Rougé P. Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. *Mol Immunol* 2008; **45**:1231-40.

78 Su M, Venkatachalam M, Teuber SS, Roux KH, Sathe SK. Impact of γ-irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. *J Sci Food Agric* 2004; **84**:1119-25.

79 Teuber S, Peterson W, Uratsu S, Dandekar A, Roux K, Sathe S. Identification and cloning of Jug r 4, a major food allergen from English walnut belonging to the legumin group. *J Allergy Clin Immunol* 2003; **111**:S248.

80 Wallowitz M, Peterson WR, Uratsu S, Comstock SS, Dandekar AM, Teuber SS. Jug r 4, a legumin group food allergen from walnut (*Juglans regia* Cv. Chandler). *J Agric Food Chem* 2006; **54**:8369-75.

81 Albillos SM, Jin T, Howard A, Zhang Y, Kothary MH, Fu T-J. Purification, crystallization and preliminary X-ray characterization of Prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin. *J Agric Food Chem* 2008; **56**:5352-8.

82 Barre A, Jacquet G, Sordet C, Culerrier R, Rougé P. Homology modelling and conformational analysis of IgE-binding epitopes of Ara h 3 and other legumin allergens with a cupin fold from tree nuts. *Mol Immunol* 2007; **44**:3243-55.

83 Robotham JM, Hoffman GG, Teuber SS *et al*. Linear IgE-epitope mapping and comparative structural homology modeling of hazelnut and English walnut 11S globulins. *Mol Immunol* 2009; **46**:2975-84.

84 Masthoff LJ, Hoff R, Verhoeckx KCM *et al*. A systematic review of the effect of thermal processing on the allergenicity of tree nuts. *Allergy* 2013; **68**:983-93.

85 Witke W. The role of profilin complexes in cell motility and other cellular processes. *Trends Cell Biol* 2004; **14**:461-9.

86 Gibbon BC, Zonia LE, Kovar DR, Hussey PJ, Staiger CJ. Pollen profilin function depends on interaction with proline-rich motifs. *Plant Cell* 1998; **10**:981-93.

87 Valenta R, Duchene M, Ebner C *et al*. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; **175**:377-85.

88 Asero R, Mistrello G, Roncarolo D *et al*. Detection of clinical markers of sensitization to profilin in patients allergic to plant-derived foods. *J Allergy Clin Immunol* 2003; **112**:427-32.

89 Sampson HA, Muñoz-Furlong A, Campbell RL *et al*. Second symposium on the definition and management of anaphylaxis: summary report - Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J Allergy Clin Immunol* 2006; **117**:391-7.

90 Bock SA, Muñoz-Furlong A, Sampson HA. Fatalities due to anaphylactic reactions to foods. *J Allergy Clin Immunol* 2001; **107**:191-3.

91 Roux KH, Teuber SS, Sathe SK. Tree nut allergens. *Int Arch Allergy Immunol* 2003; **131**:234-44.

92 Teuber S, Comstock S, Sathe S, Roux K. Tree nut allergy. *Curr Allergy Asthma Rep* 2003; **3**:54-61.

93 Crespo JF, James JM, Fernandez-Rodriguez C, Rodriguez J. Food allergy: nuts and tree nuts. *Br J Nutr* 2006; **96**(S2):S95-102.

94 Chapman MD. Allergen Nomenclature. In: Lockey RF, Ledford DK, eds. *Allergens and Allergen Immunotherapy*. 4th Edn. New York: Informa Healthcare, 2008:47-58.

95 Hansen KS, Ballmer-Weber BK, Sastre J *et al*. Component-resolved in vitro diagnosis of hazelnut allergy in Europe. *J Allergy Clin Immunol* 2009; **123**:1134-41.e1133.

96 Comstock SS, McGranahan G, Peterson WR, Teuber SS. Extensive in vitro cross-reactivity to seed storage proteins is present among walnut (*Juglans*) cultivars and species. *Clin Exp Allergy* 2004; **34**:1583-90.

97 Costa J, Oliveira MBPP, Mafra I. Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods. *Food Res Int* 2013; **51**:228-35.

98 Johnson PE, Sancho AI, Crevel RWR, Mills ENC. Detection of allergens in foods. In: Nollet LML, van Hengel AJ, eds. *Food allergens: analysis instrumentation and methods*. Boca Raton: CRC Press, Taylor & Francis Group, 2011:13-27.

99 Schubert-Ullrich P, Rudolf J, Ansari P *et al*. Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview. *Anal Bioanal Chem* 2009; **395**:69-81.

100 Diaz-Amigo C. Antibody-based detection methods: from theory to practice. In: Popping B, Diaz-Amigo C, Hoenicke K, eds. *Molecular biological and immunological techniques and applications for food chemists*, New Jersey: John Wiley & Sons, Inc., 2010:223-45.

101 Doi H, Touhata Y, Shibata H *et al*. Reliable enzyme-linked immunosorbent assay for the determination of walnut proteins in processed foods. *J Agric Food Chem* 2008; **56**:7625-30.

102 Sakai S, Adachi R, Akiyama H *et al*. Determination of walnut protein in processed foods by enzyme-linked immunosorbent assay: inter laboratory study. *J AOAC Int* 2010; **93**:1255-61.

103 Niemann L, Taylor SL, Hefle SL. Detection of walnut residues in foods using an enzyme-linked immunosorbent assay. *J Food Sci* 2009; **74**:T51-7.

104 Wang H, Li G, Wu Y, Yuan F, Chen Y. Development of an indirect competitive immunoassay for walnut protein component in food. *Food Chem* 2014; **147**:106-10.

105 Picariello G, Mamone G, Addeo F, Ferranti P. The frontiers of mass spectrometry-based techniques in food allergenomics. *J Chromatogr A* 2011; **1218**:7386-98.

106 Johnson PE, Baumgartner S, Aldick T *et al*. Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. J AOAC Int 2011; **94**:1026-33.

107 Monaci L, Visconti A. Mass spectrometry-based proteomics methods for analysis of food allergens. *Trac-Trends Anal Chem* 2009; **28**:581-91.

108 Harrer A, Egger M, Gadermaier G *et al*. Characterization of plant food allergens: an overview on physicochemical and immunological techniques. *Mol Nutr Food Res* 2010; **54**:93-112.

109 Bignardi C, Elviri L, Penna A, Careri M, Mangia A. Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *J Chromatogr A* 2010; **1217**:7579-85.

110 Bignardi C, Mattarozzi M, Penna A *et al*. A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by liquid chromatography-tandem mass spectrometry. *Food Anal Meth* 2013; **6**:1144-52.

111 Heick J, Fischer M, Kerbach S, Tamm U, Popping B. Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. *J AOAC Int* 2011, **94**:1060-68.

112 Heick J, Fischer M, Pöpping B. First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J Chromatogr A* 2011; 1218:938-43.

113 Costa J, Mafra I, Carrapatoso I, Oliveira MBPP. Almond allergens: molecular characterization, detection, and clinical relevance. *J Agric Food Chem* 2012; **60**:1337-49.

114 Wang H, Yuan F, Wu Y *et al*. Detection of allergen walnut component in food by an improved real-time PCR method. *J Food Prot* 2009; **72**:2433-5.

115 Köppel R, Velsen-Zimmerli F, Bucher T Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. *Eur Food Res Technol* 2012; **235**:843-52.

116 Brezná B, Hudecová L, Kuchta T. A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. *Eur Food Res Technol* 2006; **223**:373-7.

117 Janská V, Piknová L, Kuchta T. Relative quantification of walnuts and hazelnuts in bakery products using real-time polymerase chain reaction. *Eur Food Res Technol* 2011; **232**:1057-60.

118 Janská V, Piknová L, Kuchta T. Semi-quantitative estimation of the walnut content in fillings of bakery products using real-time polymerase chain reaction with internal standard material. *Eur Food Res Technol* 2012; **235**:1033-8.

119 Yano T, Sakai Y, Uchida K *et al*. Detection of walnut residues in processed foods by polymerase chain reaction. *Biosci Biotechnol Biochem* 2007; **71**:1793-6.

120 Ehlert A, Demmel A, Hupfer C, Busch U, Engel K-H. Simultaneous detection of DNA from 10 food allergens by ligation-dependent probe amplification. *Food Add Contam A* 2009; **26**:409-18.

121 Costa J, Oliveira MBPP, Mafra I. Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Res Int* (Doi:10.1016/j.foodres.2013.09.047).

122 Pilolli R, Monaci L, Visconti A. Advances in biosensor development based on integrating nanotechnology and applied to food-allergen management. *TrAC-Trends Anal Chem* 2013; **47**:12-26.

123 Cosnier S, Mailley P. Recent advances in DNA sensors. *Analyst* 2008; **133**:984-91.

124 Wang W, Li Y, Zhao F, Chen Y, Ge Y. Optical thin-film biochips for multiplex detection of eight allergens in food. *Food Res Int* 2011; **44**:3229-34.

125 Berin MC, Sampson HA. Food allergy: an enigmatic epidemic. *Trends Immunol* 2013; **34**:390-7.

126 Tang MLK, Martino DJ. Oral immunotherapy and tolerance induction in childhood. *Pediatr Allergy Immunol* 2013; **24**:512-20.

127 Blumchen K, Ulbricht H, Staden U *et al*. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 2010; **126**:83-91.e81.

128 Jones SM, Pons L, Roberts JL *et al*. Clinical efficacy and immune regulation with peanut oral immunotherapy. *J Allergy Clin Immunol* 2009; **124**:292-300.e297.

EXPERIMENTAL PART

Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes

Food Research International, (DOI: 10.1016/j.foodres.2013.09.047**)**

(DOI: 10.1016/j.foodres.2013.09.047)

Contents lists available at SciVerse ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes

Joana Costa, Maria Beatriz P.P. Oliveira, Isabel Mafra*

REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Portugal.

*Corresponding author: Tel: +351 220428640. Fax: +351 226093390. E-mail: isabel.mafra@ff.up.pt

ABSTRACT

Walnut is commonly included as an ingredient in bakery products, being widely appreciated by many consumers. However, walnut is also classified as an allergenic food, representing a potential risk for the health of sensitised individuals when present in processed foods as a hidden allergen. In the present work, we developed a single-tube nested real-time PCR system to trace walnut in bakery products and assessed the effect of heat treatment on the performance of the technique. The proposed approach, targeting the sequence encoding for the Jug r 3 allergen, enabled lowering the relative limit of detection (LOD) of the conventional real-time PCR assay from 0.005% to 0.001% of walnut in both batter and sponge cakes. The absolute LOD was 1 pg of walnut DNA (1.6 DNA copies) in both mixtures, evidencing that the performance of the method was not affected by the heat processing. To our knowledge we proposed for the first time a single-tube nested real-time PCR system as a cost-effective, robust and powerful tool for high-throughput DNA-based detection and quantification of walnut allergens in raw and processed foods.

Keywords: *Juglans regia*, walnut detection, real-time PCR, food allergens, sponge cakes.

INTRODUCTION

Food-induced allergy is, by definition, an abnormal health effect arising from a specific immunological response that occurs reproducibly on exposure to a given food (Boyce et al., 2010). In theory, any food is susceptible of triggering an allergic reaction in sensitised individuals, however about 90% of the total adverse immunological responses are specifically attributed to eight groups of foods (tree nuts, peanuts, soybean, cereals containing gluten, eggs, milk, crustaceans and fish).

Walnut (*Juglans regia*) is included in the tree nut group for which clinical symptoms have been commonly described as mild to potentially fatal adverse reactions (anaphylaxis). Among the tree nuts, walnut is frequently reported as having the highest incidence of reports inducing severe allergic reactions (Fleischer, Conover-Walker, Matsui, &Wood, 2005; Sicherer, Furlong, Muñoz-Furlong, Burks, & Sampson, 2001). In contrast to other foods such as eggs or milk, patients diagnosed with allergies to one or more tree nuts are predicted to suffer from "life persisting" allergy (Sampson, 2003).

The true prevalence of food allergies has been very difficult to establish since the majority of the information about their prevalence is based on self-reported reactions to foods (questionnaires/surveys), rather than using objective assessments as open and double-blind food challenge tests, or determined sensitisation to foods by serum immunoglobulin E (IgE) and skin prick tests (Zuidmeer et al., 2008). In Europe, food induced allergies related to tree nuts are often common, with hazelnut being reported as a major contributor, whereas in USA, allergies caused by walnut, almond and cashew consumption seem to be more frequent (Ortolani et al., 2000; Sicherer, Muñoz-Furlong, & Sampson, 2003). The precise number of patients with diagnosis of allergy to walnut or other foods is still unknown, although on the basis of a recent study encompassing several western countries, sera from test subjects were scanned for 5 allergen mixes (including walnut) from a total of 24 foods previously defined as priorities. In the referred study, walnut allergy was estimated to an overall incidence of 2.2% among the tested allergic population (Burney, Summers, Chinn, Hooper, Van Ree, & Lidholm, 2010). So far, the only proven preventive treatment for these individuals consists of the elimination of the food allergen and all the cross-reacting allergens from diet. In this sense, when choosing a food product, the allergic patients have to rely on labelling information, especially in the case of processed foods. Once the total avoidance of the allergenic food is almost impossible to accomplish, sensitised individuals may still be at risk of suffering from allergic reactions due to the presence of hidden allergens in foods owing to incorrect labelling or cross-contaminations during food processing. Therefore, to verify labelling compliance (Directive 2007/68/EC, Regulation (EU) No 1169/2011), to help the industrial management of food allergens and to ensure consumer's safety, the development of proper and highly sensitive analytical methodologies is of utmost importance (Costa, Oliveira, & Mafra, 2013).

Most of the available analytical tools rely on protein- and/or DNA-based methods for food allergen assessment (Costa, Mafra, Carrapatoso, & Oliveira, 2012a). Regarding the specific detection of walnut in raw and processed foods, some techniques have been developed targeting the allergenic/marker proteins or the DNA sequences encoding them. Among the protein-based methods, the immunochemical assays such as enzyme-linked immunosorbent assays (ELISA) (Doi et al., 2008; Niemann, Taylor, & Hefle, 2009; Sakai et al., 2010) and more recently, the mass spectrometry analysis (Bignardi, Elviri, Penna, Careri, & Mangia, 2010; Bignardi et al., 2013; Heick, Fischer, Kerbach, Tamm, & Popping, 2011) have been effectively applied for the detection and quantification of walnut in a wide range of food products. As very reliable alternatives, polymerase chain reaction (PCR) techniques have also been successfully employed to target walnut in processed foods (Brezná, Hudecová, & Kuchta, 2006; Wang et al., 2009; Wang, Li, Zhao, Chen, & Ge, 2011; Koppel, Velsen-Zimmerli, & Bucher, 2012). Until now, the few reported qualitative and quantitative PCR methods regarding walnut detection target the amplification of the gene encoding the allergenic protein Jug r 2 (Brezná et al., 2006; Wang et al., 2009; Wang et al., 2011; Koppel et al., 2012).

In this work, we proposed a new molecular method targeting a different gene encoding the allergenic protein Jug r 3 of walnut. Jug r 3 belongs to the lipid transfer proteins (LTP), being classified as a major allergen with severe clinical presentation (systemic reactions) among the walnut allergic individuals (Pastorello et al., 2004). The suggested novel approach is based on the single-tube nested real-time PCR system following its successful application to other tree nuts, namely almond (Costa et al., 2013) and hazelnut (Costa, Mafra, Kuchta, & Oliveira 2012b). Considering that walnut is often incorporated in bakery products, it was also important to exploit the effect of thermal processing on walnut detection. In general, it is well established that thermal processing can lead to conformational changes of proteins, which can affect their IgE-binding epitopes (Sathe and Sharma, 2009). Regarding the effect of food processing on DNA molecules, few information is still available although recent literature seem to indicate a negative effect on DNA detection after severe heat treatment of food (Gryson, 2010; Iniesto et al., 2013; Platteau, De Loose, De Meulenaer, & Taverniers, 2011; Scaravelli, Brohée, Marchelli, & van Hengel, 2009). Using model batter and sponge cakes, this study intended to further evaluate the influence of thermal treatment on the performance parameters of the developed conventional and single-tube nested real-time PCR systems.

MATERIALS AND METHODS

Plant foods

Walnut cultivars used in this study were acquired at Portuguese markets, including different geographical origins (USA, France, Chile, Portugal and Spain). A total of 10 different cultivars of walnut, namely “Payne”, “Vina”, “Hartley”, “Lara”, “Chandler”, “Corne”, “Franquette”, “Serr”, “Fernor” and “Marbot” were tested.

Other plant foods, totalising 31 different species, namely peanuts and tree nuts (almond, pine nut, macadamia nut, hazelnut, Brazil nut, chestnut, cashew, pistachio and pecan nut), and the plant species (wheat, soybean, lupine, fava bean, maize, oat, rye, barley, rice, pumpkin seeds, rapeseed, sunflower, tomato, peach, nectarine, apricot, plum, cherry, strawberry, blackberry and raspberry) were purchased at local markets.

Preparation of model mixtures of batter and sponge cakes spiked with walnut

Model mixtures of batter and sponge cakes were prepared containing 50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% of walnut. A basic sponge cake recipe was used containing 3 eggs, 100 g of sugar and 100 g of wheat flour with baking powder. The first spiked batter, containing 50% of walnut, was prepared by adding 200 g of grounded walnut to 200 g of batter. All the following spiking levels of walnut were obtained by serial dilution with batter, i.e., by successive additions of batter until the proportion of 0.0001% of walnut.

All procedures were performed similarly to the preparation of cakes in bakeries, being each batter blended using a cake mixer (Tefal Kitchen Machine, model QA401, SEB group, Berkshire, UK) to ensure the correct homogeneity of the model mixtures. According to the basic recipe for sponge cake preparation, eggs and sugar were first mixed for 5 min to allow formation of a creamy mixture. The wheat flour containing baking powder was then added to the mixture in small portions with continuous mixing for 5 min. After adding the grounded walnut, batter was mixed for more 3 min. From each spiked batter, a sample was collected and immediately stored at -20 ºC to minimise enzymatic activity until analysis. The remaining batter (approximately 350 g) was then baked at 200 ºC for 20 min. After cooling, a sample of each sponge cake was retrieved. Portions of the model cakes (~100 g) and other plant foods were ground and homogenised separately, into a fine powder of approximately 0.3 mm of diameter in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany) using different containers and material, previously treated with a DNA decontamination solution. The fruits, namely, tomato, peach, apricot, plum and cherry, were lyophilised before grinding. To avoid accidental cross-contamination among samples, plant foods and standards, all materials were grounded in different days. After grinding, the samples were immediately stored at -20 ºC until DNA extraction.

DNA extraction

DNA was extracted from 200 mg of sample using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations as described by Costa et al. (2012b).

Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

Target gene selection and oligonucleotide primers

The DNA sequence corresponding to *Juglans regia*, nonspecific LTP mRNA, encoding for the Jug r 3 allergen was retrieved from the NCBI database (accession no. EU780670.1). For the development of single-tube nested real-time PCR system, two sets of primers with different annealing temperatures (T_a) were designed using the software Primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). The defined parameters for the design of primers were set considering a difference higher than 10 ºC in the annealing temperatures between the two sets of primers (Costa et al., 2012b; Costa et al., 2013). Thus, the first pair of primers (Jug3F/Jug3R) was designed with an optimal T_a of 66 ºC, while the second (Jug3FN/Jug3RN) has a T_a of 54 ºC. For the application of real-time PCR systems a hydrolysis probe (Jug3P) was also designed (Table 1). To confirm the *in silico* specificity of the designed primers, the basic local alignment search tool BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify regions of local similarity between the selected nucleotide sequence and homologue sequences of different species.

Sequencing

For sequencing purposes, a third set of primers (Jug3FS/Jug3RS) was specifically designed to produce a larger PCR fragment (319 bp), encompassing the target region of 136 bp amplified by the primers Jug3F/Jug3R, using the end-point PCR conditions described below. Therefore, the amplified fragments of ten different walnut cultivars were sequenced in a specialised research facility (STABVIDA, Lisbon, Portugal). Before sequencing, all PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality.

Table 1. Key data of primers and probe designed to target *Juglans regia*, nonspecific LTP protein mRNA, encoding for Jug r 3 allergen (NCBI accession no. EU780670.1)

Oligonucleotides	Sequence (5'-3')	T_a (ºC)	Amplicon (bp)
Outer primers			
Jug3F	TAC GGT TCC TAC AGT CCC TCC AA	62.4	136
Jug3R	AGG GTT GAG TCC GGG GAT GGA A	64.0	
Inner primers			
Jug3FN	CTC CAA GCT GCT GCA AT	52.8	99
Jug3RN	AAC CAG AAG TCT TTT TCA G	50.2	
Sequencing primers			
Jug3FS	TCA GGC ATG GTG CTG CTG TGT ATG	64.4	319
Jug3RS	TGC AGT TAG TGG AGG TGC TGA TCT	62.7	
Probe			
Jug3P	FAM-TCA ACA AAG CGG CCG CTA CCA CAG CT-BHQ1	68.0	

To evaluate the quality of the extracted DNA, all extracts were amplified by end-point PCR using universal eukaryotic primers (18SRG-F – CTGCCCTATCAACTTTCGATGGTA and 18SRG-R – TTGGATGTGGTAGCCGTTTCTCA), targeting a conserved region of 18S ribosomal RNA gene (NCBI accession no. HQ873432.1).

Primers and probe were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

End-point PCR

For sequencing, PCR products were obtained using a 25 µL of total reaction volume containing 2 µL of walnut DNA extract (100 ng), 670 mM of Tris–HCl (pH 8.8), 160 mM of $(NH_4)_2SO_4$, 0.1% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of $MgCl_2$ and 240 nM of each primer Jug3FS/Jug3RS (Table 1). The assays were carried out in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA), according to the following programme: initial denaturation at 95 ºC for 5 min; 40 cycles at 95 ºC for 30 s, 65 ºC for 45 s and 72 ºC for 1 min; with a final extension at 72 ºC for 5 min.

Universal PCR products were obtained using the same reaction components as above, but adding 240 nM of 18SRG-F/18SRG-R. The assays were performed according to the following programme of temperatures: initial denaturation at 95 ºC for 5 min; 33 cycles at 95 ºC for 30 s, 65 ºC for 30 s and 72 ºC for 30 s; with a final extension at 72 ºC for 5 min.

Conventional and single-tube nested real-time PCR systems

The conventional real-time PCR assays were performed in 20 µL of total reaction volume, containing 2 µL of DNA (100 ng), 1x of SsoFast Probes Supermix (Bio-Rad, Hercules, CA, USA), 300 nM of each outer primer Jug3F/Jug3R and 200 nM of hydrolysis probe Jug3P (Table 1). For single-tube nested real-time PCR amplification, the mix was similar with the addition 300 nM of inner primers Jug3FN/Jug3RN that were specifically designed for this assay (Table 1). All real-time PCR assays were carried out on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). The conventional real-time PCR amplifications were performed according to the following temperature programme: 95 ºC for 5 min, 50 cycles at 95 ºC for 15 s and 66 ºC for 45 s, with the collection of fluorescence signal at the end of each cycle. The assays of the single-tube nested real-time PCR were done in two different phases: (1) 95 ºC for 5 min, 10 cycles at 95 ºC for 15 s and 66 ºC for 45 s, without collecting the fluorescence signal; (2) 40 cycles at 95 ºC for 15 s, 54 ºC for 20 s and 72 ºC for 30 s, with the collection of fluorescence signal at the end of each cycle. Data were processed and analysed using the software Bio-Rad CFX Manager 3.0 (Bio-Rad, Hercules, CA, USA). Cycle threshold (Ct) values were calculated using the software at automatic threshold setting. Conventional real-time PCR and nested real-time PCR trials were repeated in two independent assay using eight replicates in each one.

Statistical analysis

The independent samples t-test with the statistical programme IBM SPSS STATISTICS (21.0 package, IBM Corporation, New York, USA) was performed to evaluate the significance of differences between the Ct values of batter and sponge cakes at the same spiking level. All data were previously assessed for normality by Shapiro-Wilk test. Significant differences were considered when $p < 0.05$.

RESULTS

In this study, for the specific detection of walnut in bakery products, the *Juglans regia* nonspecific LTP mRNA, encoding for the Jug r 3 allergen was retrieved from the NCBI database. Based on the recent and successful application of the single-tube nested real-time PCR system to hazelnut (Costa et al., 2012b) and almond (Costa et al., 2013), this novel approach was also employed for the detection and quantification of trace amounts of DNA walnut allergen (Jug r 3) in bakery products. Owing to the fact that bakery products are consumed after being submitted to extensive food processing such as thermal treatments, it was considered essential to evaluate its effect on the detection of walnut DNA using the new proposed method.

Sequencing of PCR products

The expected products of real-time PCR systems were designed to have a length of 136 bp, which is considered a rather small fragment for direct sequencing. To overcome this problem, an easy, reliable and cost-effective strategy consisted on sequencing a larger fragment of 319 bp to allow an accurate sequencing of the contained target region of 136 bp.

The sequencing results of the ten walnut cultivars presented high resolution electropherograms, therefore confirming the adequacy of the chosen strategy. The method allowed sequencing the fragments in all the extension of the target region (Fig. 1), with no differences encountered for almost all the tested walnut varieties, with the exception of Marbot, Corne and Serr cultivars. In these three cases, a single nucleotide difference regarding the presence of a C nucleotide instead of the expected T in position 245 can be observed (Fig. 1). However, the specificity of the chosen target region was not affected by this small difference among cultivars, since all cultivars exhibited the same performance by PCR techniques. The sequenced fragments were also aligned with the *Juglans regia,* nonspecific LTP mRNA sequence from GenBank, exhibiting ~100% homology.

Specificity

To guarantee the absence of any possible false-negative, the DNA extracts from all plant foods and model mixtures were previously evaluated for their amplifiability using the primers 18SRG-F/18SRG-R targeting an universal eukaryotic fragment. All tested samples and model mixtures amplified positively for the expected product of 113 bp, confirming that the DNA extracts presented the adequate quality and purity for PCR amplification. In addition to *in silico* analysis, the specificity of the proposed primers for walnut identification was determined experimentally. The specificity was tested using different plant species to evaluate any possible cross-reactivity. From the 31 species investigated, there was no observable cross-reactivity with the designed primers, evidencing the adequate specificity of the assay for the correct identification of walnut.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       61         71         81         91        121        121        131
EU780670.1      TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Hartley         TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Chandler        TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Lara            TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Franquette      TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Fernor          TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Marbot          TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Vina            TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Corne           TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Payne           TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG
Serr            TTGCAGAGGC GGTCATAACA TGTGGGCAGG TGGCTAGCAG CGTGGGGAGT TGCATTGGCT ACCTCAGGGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      141        151        161        171        181        191        201
EU780670.1      TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Hartley         TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Chandler        TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Lara            TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Franquette      TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Fernor          TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Marbot          TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Vina            TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Corne           TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Payne           TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA
Serr            TACGGTTCCT ACAGTCCCTC CAAGCTGCTG CAATGGGGTC AAGAGCCTCA ACAAAGCGGC CGCTACCACA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      211        221        231        241        251        261        271
EU780670.1      GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Hartley         GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Chandler        GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Lara            GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Franquette      GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Fernor          GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Marbot          GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACCT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Vina            GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Corne           GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACCT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Payne           GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACTT CTGGTTCCAT CCCCGGACTC AACCCTGGTC
Serr            GCTGACCGCC AGGCCGCCTG TGAGTGCCTG AAAAAGACCT CTGGTTCCAT CCCCGGACTC AACCCTGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      281        291        301        311        321        331
EU780670.1      TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Hartley         TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Chandler        TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Lara            TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Franquette      TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Fernor          TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Marbot          TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Vina            TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Corne           TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Payne           TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
Serr            TTGCTGCTGG CCTCCCAGGC AAATGTGGTG TCAGTGTTCC TTACAAGATC AGCACCTC
```

Fig. 1. Alignment of PCR products of 10 walnut cultivars obtained by sequencing. The shadowed region of 136 bp corresponds to the PCR fragments using "outer" primers Jug3F/Jug3R. In position 245, a difference in nucleotide C instead of a T is highlighted for three cultivars (Marbot, Corne and Serr).

Development of analytical method

The real-time PCR systems (conventional and single-tube nested real-time PCR) were optimised using model mixtures of batter and sponge cakes spiked with known amounts of walnut. For the comparison and the evaluation of the proposed real-time PCR systems, the prerequisites for method performance established in the available documents of MIQE guidelines (Bustin et al., 2009) and of the definition of minimum performance requirements for analytical methods of genetically modified organisms testing (Mazzara et al., 2008) were carefully considered.

Real-time PCR system

The real-time PCR assays were performed using model mixtures of batter and sponge cakes spiked with walnut ranging from 50% to 0.0001%. The amplification results show the detection of walnut in batter and sponge cakes down to 0.001% (10 mg/kg), but only in 4 and 6 out of 16 replicates, respectively (Table 2). Considering that the limit of detection (LOD) is the lowest concentration level with positive identification of the analyte at least in 95% of the times (Bustin et al., 2009; Mazzara et al., 2008), in the present work the LOD was defined when amplification was positive for the total number of replicates. Therefore, the relative LOD determined for both batter and sponge cakes corresponded to 0.005% (50 mg/kg) of walnut since it was the lowest level with positive amplification of all replicates (Table 2, Fig. 2A,B). The relative limit of quantification (LOQ) achieved with the conventional real-time PCR system was equal to the LOD (50 mg/kg of walnut in batter and sponge cakes) since it was within the linear range of the calibration curve.

To evaluate the performance of the real-time PCR method, several parameters have to comply with the acceptance criteria established for this type of assay. Accordingly, the correlation coefficient (R^2) should be above 0.98, the PCR efficiency should range between 90%-110% and the slope between -3.6 and -3.1 (Bustin et al., 2009; Mazzara et al., 2008). In the performed real-time PCR runs the values for R^2, PCR efficiency and slope were in good agreement with the acceptance criteria. In the case of model mixtures of walnut in batter, mean values for R^2, PCR efficiency and slope were 0.9939, 109.8% and -3.1072, respectively (Table 2, Fig. 2A).

For the real-time PCR assays carried out with model walnut cakes, mean values for R^2, PCR efficiency and slope were 0.9964, 106.6% and -3.1724, respectively (Table 2, Fig. 2B). The Ct mean values at the LOD (50 mg/kg) obtained by both model mixtures of walnut in batter and sponge cakes were 35.95 and 35.38, respectively, presenting a difference of approximately 0.6 cycles that could also be observed for other spiked levels

(Table 2). In spite of the variances in Ct values, statistical significant differences ($p < 0.05$) were only found for spiked levels of 5% and 10% of walnut between batter and cake.

The dynamic range and the absolute sensitivity of the real-time PCR assay were established using the extracts of model mixtures of batter and sponge cake spiked with 50% of walnut, which were 10-fold serially diluted to cover 6 orders of magnitude of the analyte (100 ng to 1 pg of walnut).

Table 2. Real-time PCR and single-tube nested real-time PCR results for the relative detection of spiked walnut in batter and sponge cakes.

	Conventional Real-time PCR		Single-tube nested real-time PCR	
	Batter	Sponge cakes	Batter	Sponge cakes
Spiked level (%)	Ct ± SD [a]	Ct ± SD	Ct ± SD	Ct ± SD
0	nd [b]	nd	nd	nd
0.0001	nd	nd	nd	nd
0.0005	nd	nd	27.86 ± 0.51 (8/16)	28.26 ± 1.04 (8/16)
0.001	37.40 ± 0.29 (4/16) [c]	36.71 ± 0.87 (6/16) [c]	27.70 ± 0.86 (16/16)	27.85 ± 0.99 (16/16)
0.005	35.95 ± 0.79 (16/16)	35.38 ± 0.86 (16/16)	26.31 ± 0.85 (16/16)	25.28 ± 0.94 (16/16)
0.01	34.75 ± 0.54 (16/16)	34.85 ± 0.47 (16/16)	24.90 ± 0.88 (16/16)	24.50 ± 0.65 (16/16)
0.05	32.99 ± 0.36 (16/16)	32.60 ± 0.38 (16/16)	22.86 ± 0.46 (16/16)	22.29 ± 0.31 (16/16)
0.1	31.63 ± 0.30 (16/16)	31.25 ± 0.29 (16/16)	21.61 ± 0.29 (16/16)	20.93 ± 0.47 (16/16)
0.5	29.21 ± 0.32 (16/16)	29.18 ± 0.23 (16/16)	19.37 ± 0.22 (16/16)	18.86 ± 0.27 (16/16)
1.0	28.41 ± 0.15 (16/16)	28.46 ± 0.23 (16/16)	18.40 ± 0.21 (16/16)	18.72 ± 0.31 (16/16)
5.0	26.17 ± 0.12 (16/16) [d]	26.01 ± 0.22 (16/16)	16.28 ± 0.13 (16/16) [f]	16.03 ± 0.33 (16/16)
10	25.18 ± 0.15 (16/16) [e]	24.61 ± 0.32 (16/16)	15.33 ± 0.23 (16/16)	14.71 ± 0.30 (16/16)
50	23.97 ± 0.24 (16/16)	23.21 ± 0.16 (16/16)	13.80 ± 0.25 (16/16) [g]	13.13 ± 0.16 (16/16)
Correlation coefficient (R^2)	0.9939	0.9964	0.9962	0.9962
Slope	-3.1072	-3.1724	-3.1236	-3.1351
PCR efficiency (%)	109.8	106.6	109.0	108.4

[a] Mean cycle threshold (Ct) values ± standard deviation (SD). [b] nd, not detected. [c] (positive amplifications/total replicates). [d,e,f,g] significant differences at $p < 0.05$ (t-test) between Ct values of batter and sponge cake at the same spiking level

The obtained data show that walnut DNA was amplified until a dilution factor of 10,000, with an absolute LOD of 10 pg for both calibration curves (batter and sponge cakes), corresponding to approximately 16 genomic DNA copies (Table 3, Fig. 3A,B). The copy number was determined according to the available walnut genome size (0.62 pg) retrieved from the Plant DNA C-values database (RBG, Kew), assuming that the targeted sequences are single copy genes. In the case of cakes, the real number of walnut genomic copies detected with this system could be slightly lower considering that some loss of genomic material could have occurred during thermal processing.

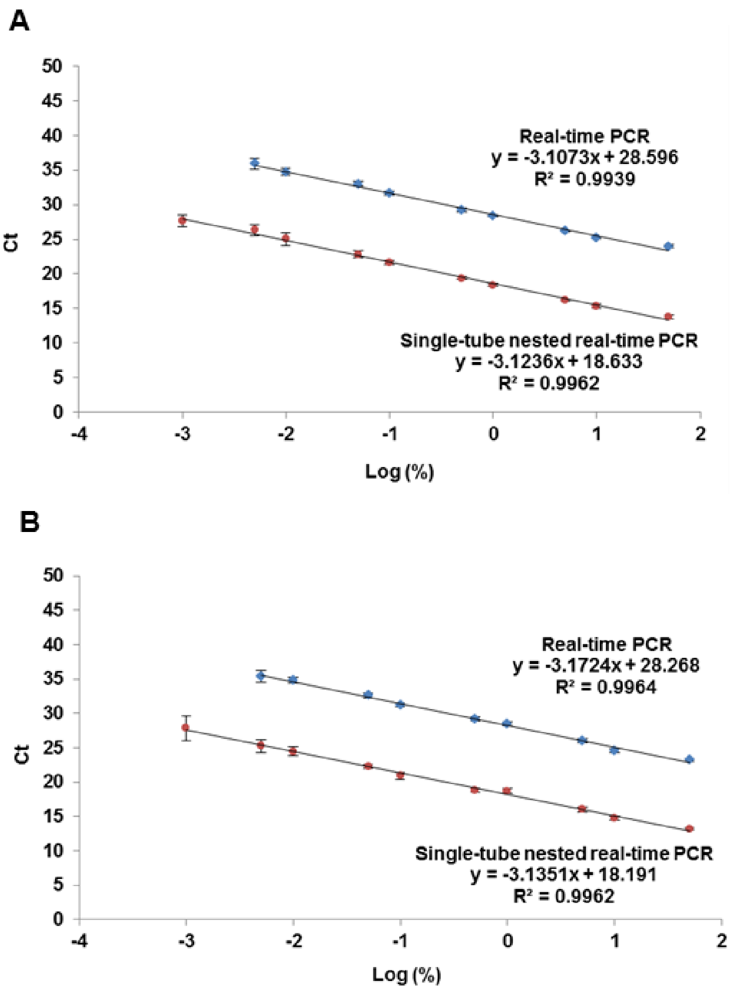


Fig. 2. Comparison of calibration curves obtained with conventional real-time PCR and single-tube nested real-time PCR of model mixtures containing 50% to 0.001% of walnut in batter (A) and sponge cakes (B). Mean values and corresponding standard deviations of *n*=16 replicates.

The Ct mean values at the absolute LOD (10 pg) obtained by real-time PCR runs with the model mixtures of walnut in batter and sponge cake were 36.89 and 35.80, respectively, presenting a difference of approximately 1 cycle that could also be observed for other spiked levels (Table 3). However, variations in Ct values reflected statistical significant differences ($p < 0.05$) only for the absolute walnut DNA of 1,000 pg between batter and cake.

All the real-time PCR assays of serially diluted extracts exhibited high performance, with mean values of 96.6% and 97.8% for PCR efficiency, slope of -3.4067 and -3.3766 and corresponding correlation coefficients of 0.9995 and 0.9993, for the calibration curves obtained from the amplification of walnut in batter (Table 3, Fig. 3A) or in sponge cakes (Table 3, Fig. 3B), respectively. In general and for both cases of relative and absolute quantification, DNA extracts from model mixtures of walnut in cakes started to amplify earlier than the corresponded level of walnut in batter.

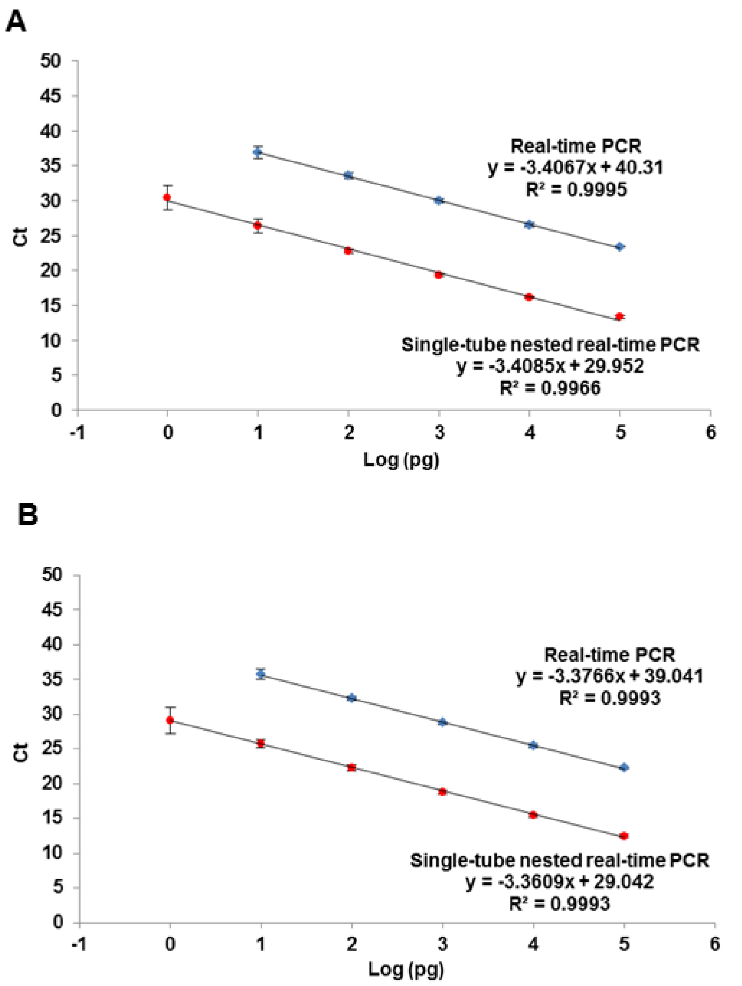


Fig. 3. Comparison of calibration curves obtained with conventional real-time PCR and single-tube nested real-time PCR of walnut DNA 10-fold serially diluted (100 ng to 1 pg) from 50% model mixture of walnut in batter (A) and sponge cake (B). Mean values and corresponding standard deviations of *n*=16 replicates.

Single-tube nested real-time PCR system

The development of the single-tube nested real-time PCR system for the detection Jug r 3 gene was based on the use of DNA extracts of model mixtures of batter and sponge cakes spiked with 50% down to 0.0001% of walnut, similarly to the conventional system. The application of this new approach allowed decreasing the LOD from 0.005% (50 mg/kg) to 0.001% (10 mg/kg) for walnut in batter and sponge cakes, respectively, which were 5× lower than the same values determined with the conventional real-time PCR system (Table 2, Fig. 2A,B).

Table 3. Real-time PCR and single-tube nested real-time PCR results for the absolute detection of walnut DNA extracted from model mixtures of 50% of walnut in batter and in sponge cake serially diluted (10-fold) ranging from 100 ng to 1 pg.

	Conventional Real-time PCR				Single-tube nested Real-time PCR			
	50% of walnut in cake batter		50% of walnut in sponge cake		50% of walnut in cake batter		50% of walnut in sponge cake	
Absolute quantity (pg)	Ct ± SD [a]	DNA copies [b]	Ct ± SD	DNA copies	Ct ± SD [a]	DNA copies	Ct ± SD	DNA copies
1	40.33 ± 2.00 (6/16) [c]	1.6	40.70 ± 2.51 (6/16)	1.6	30.41 ± 0.98 (16/16)	1.6	29.10 ± 1.00 (16/16)	1.6
10	36.89 ± 0.86 (16/16)	16	35.80 ± 0.74 (16/16)	16	26.42 ± 0.96 (16/16) [e]	16	25.75 ± 0.57 (16/16)	16
100	33.62 ± 0.47 (16/16)	161	32.25 ± 0.32 (16/16)	161	22.81 ± 0.27 (16/16)	161	22.31 ± 0.41 (16/16)	161
1,000	30.02 ± 0.32 (16/16) [d]	1613	28.74 ± 0.20 (16/16)	1613	19.40 ± 0.17 (16/16)	1613	18.79 ± 0.23 (16/16)	1613
10,000	26.53 ± 0.25 (16/16)	16129	25.44 ± 0.28 (16/16)	16129	16.15 ± 0.11 (16/16)	16129	15.40 ± 0.17 (16/16)	16129
100,000	23.40 ± 0.09 (16/16)	161290	22.32 ± 0.16 (16/16)	161290	13.40 ± 0.20 (16/16)	161290	12.48 ± 0.23 (16/16)	161290
Correlation coefficient (R^2)	0.9995		0.9993		0.9966		0.9993	
Slope	-3.4067		-3.3766		-3.4085		-3.3609	
PCR efficiency (%)	96.6		97.8		96.5		98.4	

[a] Mean cycle threshold (Ct) values ± standard deviation (SD). [b] Number of walnut haploid genome copies (0.62 pg) (RBG, Kiew).

[c] (positive amplifications/total replicates). [d, e] significant differences at $p < 0.05$ (t-test) between Ct values of batter and sponge cake at the same spiking level

Additionally, with the nested approach it was also possible to amplify walnut DNA down to 0.0005% (5 mg/kg), but only for half the replicates, not being considered for this reason as the LOD. Within the linear range of the calibration curve, the LOD was also considered the LOQ for this system. All the single-tube nested real-time PCR runs were in good compliance with the acceptance criteria for real-time PCR parameters, presenting mean values for R^2 of 0.9962 and 0.9962, slopes of -3.1236 and -3.1351 and PCR efficiencies of 109.0% and 108.4%, using the model mixtures of batter (Table 2, Fig. 2A) and sponge cakes (Table 2, Fig. 2B), respectively.

The same dynamic range was used to evaluate the novel system aiming at establishing the absolute LOD. Thus, model mixtures of batter and sponge cake spiked with 50% of walnut were 10-fold serially diluted ranging from 100 ng to 1 pg of walnut. Single-tube nested real-time PCR assays presented positive amplification for all the replicates used over the entire dynamic range, covering six orders of magnitude and allowing the absolute detection of walnut until a dilution factor of 100,000. The absolute LOD was 10x lower than the conventional system, enabling amplification of walnut DNA down to 1 pg, in both calibration curves (Table 3, Fig. 3A,B). This value of detection corresponds to approximately 1.6 genomic copies of walnut, evidencing the high sensitivity of the developed method. Like for the conventional real-time PCR system, the real value of sensitivity in cakes could be slightly lower due to the probable loss of genomic material during thermal treatment. Considering that the lowest amplified level was within the linear range of the calibration curve, the LOQ was the same value of the LOD. The performance parameters of single-tube nested real-time PCR assays were also in accordance with the required criteria, presenting mean values for PCR efficiency of 96.5% and 98.4%, slopes of -3.4085 and -3.3609 and correlation coefficients of 0.9966 and 0.9993, for model mixtures of batter (Table 3, Fig. 3A) and sponge cakes (Table 3, Fig. 3B), respectively. As previously noted, with the novel nested real-time PCR system the DNA extracts of walnut in sponge cakes started to amplify earlier than the correspondent level of walnut in batter. As previously highlighted, differences in Ct values between batter and sponge cakes were generally not statistically significant. For the relative detection of walnut, significant differences ($p < 0.05$) were only noted for the levels of 5% and 50%, while in the case of absolute detection it occurred in the level of 10 pg.

DISCUSSION

The preparation of foods such as bakery products involves procedures that may alter the native structure of several components. During cooking, processes such as thermal treatments (boiling, roasting, baking), partial hydrolysis, pH variations, high pressure processing and/or radiation are known to affect the integrity of proteins (Mills, Sancho, Moreno, & Kostyra, 2007). This represents one of the major problems associated with the detection of allergenic proteins in processed foods by immunological methods (Cucu, Platteau, Taverniers, Devreese, De Loose, & De Meulenaer, 2011; Cucu, Platteau, Taverniers, Devreese, De Loose, & De Meulenaer, 2013; Platteau et al., 2011). On the other hand, the elevated stability of DNA upon industrial processing has elected this molecule as a favoured target for allergen evaluation (Costa, Mafra, Carrapatoso, & Oliveira, accepted). Therefore, DNA-based methods have been regarded as powerful alternatives for the detection and identification of allergenic food sources in processed foods. Although the higher stability of DNA molecules upon food processing when compared with proteins, they can still be negatively affected by cooking processes such as baking roasting and autoclaving. In this regard, some studies describing the influence of thermal treatment on hazelnut and peanut DNA detection by real-time PCR technique have been reported (Iniesto et al., 2013; Platteau, et al., 2011; Scaravelli et al., 2009). Plateau et al. (2011) referred that baking affected the sensitivity of a real-time PCR assay targeting Cor a 8 allergen encoding gene for the identification of hazelnut in cookies. In that study, the sensitivity achieved was 100 mg/kg of hazelnut in dough and 1000 mg/kg of hazelnut in cookies, evidencing a reduced sensitivity after heat treatment. Iniesto et al. (2013) also evaluated the effect of different food processing such as roasting, autoclaving and high pressure treatments to raw hazelnut on DNA yield and amplifiability. The results indicated that roasting and autoclaving reduced the ability to detect hazelnut DNA, which was supported by the low DNA yields and elevated Ct values for the thermal treated samples. Scaravelli et al. (2009) reported an increasing inaccuracy when detecting low levels of peanut DNA (10 mg/kg) in cookies by a real-time PCR assay, as a consequence of prolonging the time of baking.

The described negative effect of heat treatments on the detection of walnut DNA was not verified with both the real-time PCR and single-tube nested real-time PCR systems. In the case of the model sponge cakes with walnut, heat treatment seems to stabilise those samples. It is important to refer that all cakes were baked using the same combination of temperature (200 ºC) and time (20 min), which is commonly used for baking cakes at industrial level. Thus, in order to determine the potentially negative effect on DNA detection using the proposed systems, other temperatures or increasing baking periods should be further investigated. In this study, a basic sponge cake was used to prepare the model mixtures of cakes spiked with walnut. The fact of using fresh eggs instead of lyophilised could have also contributed to the strong enzymatic activity of the batter. In spite of the immediate preservation of the batter mixtures at -20 ºC, that could not be sufficient to prevent some potential DNA degradation as consequence of the enzymatic activity (Gryson, 2010). All these facts could be considered probable reasons for the herein results concerning the same LOD and LOQ for both processed and unprocessed cakes. Another important issue to emphasise is related to the choice of small walnut amplicons, which enables amplification of highly degraded DNA as a result of heat processing.

From the few reports in literature concerning the specific detection and quantification of walnut, the sensitivity levels for the developed methods based on conventional TaqMan real-time PCR assays ranged from 10-100 mg/kg of Jug r 2 DNA in incurred foods (Brezná et al., 2006; Koppel et al., 2012; Wang et al., 2009). Therefore, the obtained LOD of 50 mg/kg of walnut by means of the proposed real-time PCR system targeting the gene encoding the Jug r 3 allergen is in good agreement with those reports.

The development of a novel approach based on single-tube nested real-time PCR system was successfully achieved in the detection of walnut in cakes, as previously succeeded in the cases of hazelnut (Costa et al., 2012b) and almond (Costa et al., 2013). Similarly to hazelnut and almond, the sensitivity was increased with the nested system since the relative LOD had a 5-fold reduction down to 10 mg/kg of walnut in batter and sponge cakes. In terms of absolute detection, the present nested system allowed amplifying walnut DNA down to 1 pg, representing approximately 1.6 DNA copies, which is in good agreement with the reported absolute LOD values of 1 and 3.9 DNA copies obtained by single-tube nested real-time PCR detection of hazelnut (Costa et al., 2012b) and almond (Costa et al., 2013), respectively. It is important to refer that after thermal processing some walnut genomic material could be lost as consequence of baking, suggesting that this limit of sensitivity could be lower than the value herein estimated. In this sense, the use of both genomic DNA and plasmid serial dilutions as reference for the calculation of the absolute sensitivity of the novel method could be recommended (D'Andrea, Coisson, Travaglia, Garino, & Arlorio, 2009).

After the heat treatment, sponge cakes presented the same relative and absolute LOD and LOQ of the model mixtures prepared with batter, highlighting an apparent robustness of the method. When compared to the other reports (Brezná et al., 2006; Koppel et al., 2012; Wang et al., 2009), the proposed nested real-time PCR system can be considered a technique with elevated specificity (no cross-reactivity with other plant-foods) and high sensitivity.

Presently, the number of commercial real-time PCR kits for the detection and quantification of allergens in foods is still limited. When compared to the real-time PCR commercial kit available for the detection of walnut allergens in foods, the presented systems show higher relative LOD (10-50 mg/kg) than the 0.4 mg/kg stated by SureFood® allergen walnut (r-Biopharm, Darmstadt, Germany). Regarding the absolute sensitivity, the proposed single-tube nested real-time PCR technique presented lower LOD (1.6 pg DNA copies) than the stated by the referred kit (5 DNA copies). However, this commercial real-time PCR system is not for quantification purposes since no calibration curve is performed in the assay, thus only enabling positive/negative responses until a referred limit of 0.4 mg/kg of walnut. Additionally, both the proposed real-time PCR systems for the specific detection of walnut do not present any cross-reactivity with more than 31 plant species tested, whereas the commercial kit reveal cross-reactivity with pecan nut.

CONCLUSION

In the present work, a molecular approach based on single-tube nested real-time PCR was effectively developed with demonstrated usefulness to trace minute amounts of walnut in bakery products. As expected, the proposed novel system provides advantages in terms of accuracy, sensitivity, specificity, dynamic range and high-throughput capacity over the conventional real-time PCR systems reported herein and in the literature. In addition, the performance of the single-tube nested real-time PCR system was not influenced by the heat processing since the sensitivities attained with both model mixtures of walnut in batter and sponge cakes were similar and Ct values were generally not significantly different. As a result, this novel technique could be widely applied to bakery products ensuring a LOD of 10 mg/kg, with the potential to obtain quantitative information by means of an appropriate calibration curve.

In general terms, this technology has the potential to be used with other model mixtures namely bread, cookies and other types of cakes or commercial food products, as already exploited in the case of hazelnut (Costa et al., 2012b). To our knowledge this is the first work evaluating the effect of thermal processing on the detection and quantification of DNA encoding walnut allergens by conventional and single-tube nested real-time PCR systems.

Acknowledgements

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2013 and by University of Porto “Projectos Pluridisciplinares” IJUP. Joana Costa is grateful to FCT PhD grant (SFRH/BD/64523/2009) financed by POPH-QREN (subsidised by FSE and MCTES).

REFERENCES

Bignardi, C., Elviri, L., Penna, A., Careri, M., & Mangia, A. (2010). Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods. *Journal of Chromatography A*, *1217*, 7579-7585.

Bignardi, C., Mattarozzi, M., Penna, A., Sidoli, S., Elviri, L., Careri, M., & Mangia, A. (2013). A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by Liquid Chromatography–Tandem Mass Spectrometry. *Food Analytical Methods*, *6*, 1144-1152.

Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., Plaut, M., Cooper, S. F., Fenton, M. J., and the, & NIAID-Sponsored Expert Panel. (2010). Guidelines for the diagnosis and management of food allergy in the United States: Report of the NIAID-Sponsored Expert Panel. *Journal of Allergy and Clinical Immunology*, *126*, S1-S58.

Brežná, B., Hudecová, L., & Kuchta, T. (2006). A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. *European Food Research and Technology*, *223*, 373-377.

Burney, P., Summers, C., Chinn, S., Hooper, R., Van Ree, R., & Lidholm, J. (2010). Prevalence and distribution of sensitization to foods in the European Community Respiratory Health Survey: a EuroPrevall analysis. *Allergy*, *65*, 1182-1188.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*, 611-622.

Costa, J., Mafra, I., Carrapatoso, I., & Oliveira, M. B. P. P. (2012a). Almond allergens: molecular characterization, detection, and clinical relevance. *Journal of Agricultural and Food Chemistry*, *60*, 1337-1349.

Costa, J., Mafra, I., Kuchta, T., & Oliveira, M. B. P. P. (2012b). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry*, *60*, 8103-8110.

Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods. *Food Research International*, *51*, 228-235.

Costa, J., Mafra, I., Carrapatoso, I., & Oliveira, M. B. P. P. Hazelnut allergens: molecular characterization, detection, and clinical relevance. *Critical Reviews in Food Safety and Nutrition.* (Accepted).

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., & De Meulenaer, B. (2011). ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Additives & Contaminants: Part A*, *28*, 1-10.

Cucu, T., Platteau, C., Taverniers, I., Devreese, B., De Loose, M., and De Meulenaer, B. (2013). Effect of partial hydrolysis on the hazelnut and soybean protein detectability by ELISA. *Food Control*, *30*, 497-503.

D'Andrea, M., Coisson, J. D., Travaglia, F., Garino, C., & Arlorio, M. (2009). Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (*Corylus avellana* L.) in foods through calibration via plasmid reference standard. *Journal of Agricultural and Food Chemistry*, *57*, 11201-11208.

Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC regarding certain food ingredients. *Official Journal of the European Union*, *L310*, 11-14.

Doi, H., Touhata, Y., Shibata, H., Sakai, S., Urisu, A., Akiyama, H., & Teshima, R. (2008). Reliable enzyme-linked immunosorbent assay for the determination of walnut proteins in processed foods. *Journal of Agricultural and Food Chemistry*, *56*, 7625-7630.

Fleischer, D. M., Conover-Walker, M. K., Matsui, E. C., Wood, R. A. (2005). The natural history of tree nut allergy. *Journal of Allergy and Clinical Immunology*, *116*, 1087– 1093.

Gryson, N. (2010). Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review. *Analytical and Bioanalytical Chemistry*, *396*, 2003-2022.

Heick, J., Fischer, M., Kerbach, S., Tamm, U., & Popping, B. (2011). Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. *Journal of AOAC International*, *94*, 1060-1068.

Iniesto, E., Jiménez, A., Prieto, N., Cabanillas, B., Burbano, C., Pedrosa, M. M., Rodríguez, J., Muzquiz, M., Crespo, J. F., Cuadrado, C., & Linacero, R. (2013). Real-time PCR to detect hazelnut allergen coding sequences in processed foods. *Food Chemistry*, *138*, 1976-1981.

Köppel, R., Velsen-Zimmerli, F., & Bucher, T. (2012). Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. *European Food Research and Technology*, *235*, 843-852.

Mazzara, M., Savini, C., Charles de Delobel, C., Broll, H., Damant, A., Paoletti, C., & van den Eede, G. (2008). European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing, European Commission: Brussels. Available online at: http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm (Website last accessed on July 5, 2013).

Mills, C. E., Sancho, A. I., Moreno, J., & Kostyra, H. (2007). The effects of food processing on allergens. In C. Mills, H. Wichers, & K. Hoffmann-Sommergruber, (Eds.), Managing allergens in food, (pp 117-133). Boca Raton: CRC Press.

Niemann, L., Taylor, S. L., & Hefle, S. L. (2009). Detection of walnut residues in foods using an enzyme-linked immunosorbent assay. *Journal of Food Science*, *74*, T51-T57.

Ortolani, C., Ballmer-Weber, B. K., Hansen, K. S., Ispano, M., Wüthrich, B., Bindslev-Jensen, C., Ansaloni, R., Vannucci, L., Pravettoni, V., Scibilia, J., Poulsen, L. K., & Pastorello, E. A. (2000). Hazelnut allergy: A double-blind, placebo-controlled food challenge multicenter study. *Journal of Allergy and Clinical Immunology*, *105*, 577-581.

Pastorello, E. A., Farioli, L., Pravettoni, V., Robino, A. M., Scibilia, J., Fortunato, D., Conti, A., Borgonovo, L., Bengtsson, A., & Ortolani, C. (2004). Lipid transfer protein and vicilin are important walnut allergens in patients not allergic to pollen. *Journal of Allergy and Clinical Immunology*, *114*, 908-914.

Platteau, C., De Loose, M., De Meulenaer, B., & Taverniers, I. (2011). Quantitative detection of hazelnut (*Corylus avellana*) in cookies: ELISA versus real-time PCR. *Journal of Agricultural and Food Chemistry*, *59*, 11395-11402.

RBG (Royal Botanic Gardens) Kew, Plant DNA C-values database, Surrey, Canada. Available online at: http://data.kew.org/cvalues/ (Website last accessed: July 8, 2013).

Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004, *Official Journal of the European Union*, *L304*, 18-63.

Sakai, S., Adachi, R., Akiyama, H., Teshima, R., Doi, H., Shibata, H., & Urisu, A. (2010). Determination of walnut protein in processed foods by enzyme-linked immunosorbent assay: inter laboratory study. *Journal of AOAC International*, *93*, 1255-1261.

Sampson, H. A. (2003). Anaphylaxis and emergency treatment. *Pediatrics*, *111*(6), 1601-1608.

Sathe, S. K., & Sharma, G. M. (2009). Effects of food processing on food allergens. *Molecular Nutrition & Food Research*, *53*, 970-978.

Scaravelli, E., Brohée, M., Marchelli, R., & van Hengel, A. (2009). The effect of heat treatment on the detection of peanut allergens as determined by ELISA and real-time PCR. *Analytical and Bioanalytical Chemistry*, *395*, 127-137.

Sicherer, S. H., Furlong, T. J., Muñoz-Furlong, A., Burks, A. W., & Sampson, H. A. (2001). A voluntary registry for peanut and tree nut allergy: Characteristics of the first 5149 registrants. *Journal of Allergy and Clinical Immunology*, *108*, 128-132.

Sicherer, S. H., Muñoz-Furlong, A., & Sampson, H. A. (2003). Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: A 5-year follow-up study. *Journal of Allergy and Clinical Immunology*, *112*, 1203-1207.

Wang, H., Yuan, F., Wu, Y., Yang, H., Xu, B., Liu, Z., & Chen, Y. (2009). Detection of allergen walnut component in food by an improved real-time PCR method. *Journal of Food Protection*, *72*, 2433-2435.

Wang, W., Li, Y., Zhao, F., Chen, Y., & Ge, Y. (2011). Optical thin-film biochips for multiplex detection of eight allergens in food. *Food Research International*, *44*, 3229-3234.

Zuidmeer, L., Goldhahn, K., Rona, R. J., Gislason, D., Madsen, C., Summers, C., Sodergren, E., Dahlstrom, J., Lindner, T., Sigurdardottir, S. T., McBride, D., & Keil, T. (2008). The prevalence of plant food allergies: A systematic review. *Journal of Allergy and Clinical Immunology*, *121*, 1210-1218.e1214.

FINAL REMARKS

FINAL REMARKS

So far, the management of allergens by the food industry is still far from being perfect. Either as a result of unintentional contamination of foods with allergen-containing ingredients or via cross-contamination along the production lines, most of food industries are not yet ready to provide foods free from allergenic ingredients. The lack of official methods and the absence of reference materials have been contributing for the delay on the definition of legal upper limits for the accidental presence of allergenic ingredients in foods. With respect to this, it is expected that in a near future, legislation will be more restrictive, obligating food industry to advertise in the labels the quantitative values for the potential presence of unintended allergenic ingredients in foods.

In this context, the research work developed during the course of this PhD aimed at advancing highly sensitive and specific methods for the correct detection and quantification of some tree nuts such as almond, hazelnut and walnut, as potential hidden allergenic ingredients in foods. Before highlighting the main results achieved for each of the studied tree nuts, it is important to refer that many efforts were effectively done to choose the most adequate method of DNA extraction, which is a highly critical task for the successful application of PCR-based methods. If for most of the studied food matrices the efforts are not fully described, for the case of chocolate, which is a very complex food matrix, a critical comparison and evaluation of several different DNA extraction protocols demonstrates its relevance. Data showed that Nucleospin food kit with minor adjustments revealed to be the most suitable for quantitative real-time PCR amplification applied to almond and hazelnut in chocolates.

Almond is widely consumed all over the world, mostly as an ingredient in several pastry (cakes, biscuits, cookies) and chocolate formulations. However, since almond is capable of inducing severe and life-threatening allergic reactions in sensitised individuals, its unexpected presence in foods represents a risk for these patients. Additionally, once almond allergy presents cross-reactivity with other fruits from the same botanical family (e.g. peach, apricot, and apple), the specific detection of this nut is of great interest. Having in mind this difficulty, a real-time PCR system using a high resolution melting analysis was developed for the specific identification of almond among other Rosaceae fruits, which represented a major contribution in this field of research since most of the available methods reveal strong cross-reactivity between almond and peach. Still aiming at improving sensitivity and specificity of almond detection, a novel system based on the combination of two PCR techniques, namely nested PCR and real-time PCR in a single-tube was proposed.

The system reached an adequate LOD for allergen analysis that was lower than the values reported in the literature (relative and absolute LOD of 50 mg/kg of almond in walnut and 1.28 pg of almond DNA (3.9 DNA copies), respectively), allowing discriminating almond from other *Prunus* species. The discrimination was succeeded because the substitution of one nucleotide was enough to provoke a shift of approximately 3 or 6 cycles between the amplification of almond and apricot or between almond and peach, respectively.

Hazelnut is another highly relevant allergenic nut, whose allergy is estimated to affect almost 7.2% of the western population. Because hazelnut allergy is often related to pollinosis, it is one of the most widely studied nuts. Concerning the detection and quantification of hazelnut, several protein- and DNA-based methods can be found in the literature. In comparison to them, the proposed novel technique based on single-tube nested real-time PCR presented high specificity and sensitivity, enabling to quantify down to 50 mg/kg of hazelnut in wheat material and an absolute LOD of 0.5 pg of hazelnut DNA (1 DNA copy) that was the lowest detection level ever reported for hazelnut. Its application to processed food samples was successfully demonstrated.

Walnut is classified as an important allergenic ingredient from the tree nut group known to be responsible for several of the most severe allergic reactions (anaphylactic shocks) related to food allergies. Thus, meaning that its correct detection and identification is of utmost relevance. To accomplish this goal, based on the same concept of the single-tube nested real-time PCR system, a novel technique was developed to trace walnut. Like for the previous nuts, the sensitivity levels were increased with the proposed system, allowing a relative quantification down to 10 mg/kg of walnut in sponge cakes, with an absolute LOD of 1 pg of walnut DNA (1.6 DNA copies). At the same time, the effect of thermal processing was evaluated, enabling to confirm the same sensitivity values in unprocessed walnut model mixtures (batter). Even after the baking treatment, DNA preserved the necessary integrity for amplification until a spiking level of 10 mg/kg of walnut cake.

In the present work, the development and application of the novel system of single-tube nested real-time PCR was fully demonstrated for the three nuts under studied and critically compared with the respective conventional real-time PCR systems. The comparison data of both conventional and nested systems showed that levels of detection and quantification could be decreased, in at least one order of magnitude, for the three tested nuts. This finding highlights the potentialities of this new system as a high sensitive and specific tool to trace other food allergens.

The contradictory opinion about the best target analyte (DNA vs. protein) continues to be shared by several researchers. If for some, the analysis of allergenic proteins should always be addressed, for others the indirect allergen evaluation through the analysis of

DNA encoding the allergenic proteins constitutes a very reliable alternative. In this context, protein-based methods were also developed during the course of this project aiming at tracing minute amounts of hazelnut proteins. Since hazelnut and almond are commonly used in chocolate formulations and considering that this is one of the most difficult food matrices, model chocolates containing hazelnut or almond were analysed. Accordingly, ELISA was advanced targeting hazelnut proteins in chocolates, enabling to detect and quantify down to 1 mg/kg and 50 mg/kg of hazelnut in chocolate, respectively. Using the same set of model chocolates, LC-MS/MS technique was applied to target eight hazelnut peptides with the same detection level of ELISA. However, the lowest levels of quantified hazelnut peptides in chocolates ranged from 100 to 5,000 mg/kg depending on the target peptide. In order to establish a full comparison among different methodologies applied to the same matrix, the above mentioned set of model chocolates were further used in the development of a real-time PCR assay with hydrolysis probe specific for hazelnut. The results showed hazelnut detection and quantification down to 1 mg/kg and 50 mg/kg, respectively, which was in good agreement with the global results of protein-based methods. However, it is important to highlight that in the case of ELISA the detection of hazelnut at concentrations below 50 mg/kg was affected by the interfering compounds present in chocolate matrix. Regarding real-time PCR system, below the level 50 mg/kg of hazelnut in chocolate, the amplification of the target was possible, but not reproducible for all replicates. Similar findings were verified in the case LC-MS/MS system to identify hazelnut peptides in chocolates.

From the techniques described herein, reliable sensitivities levels were found to be similar among protein- and DNA-based methods. Still when choosing a method to detect and quantify tree nuts or other allergenic foods, several conditions such as the main advantages or disadvantages of each method should be carefully considered. In terms of time and cost per analysis, the ELISA and the real-time PCR systems are the most appealing since LC-MS/MS platforms are expensive to acquire and to maintain, although they allow unequivocal identification of the target peptides. Although ELISA have been regarded as excellent tools for the rapid detection of allergenic ingredients in foods, the results attained with those systems should be further confirmed using different technology such as real-time PCR or LC-MS/MS, since both allow unequivocal identification of the target. With this work, it was possible to establish several of the potentialities of the real-time PCR systems as highly sensitive, specific and cost-effective methodology for the detection and quantification of food allergens.

The correct management of allergenic ingredients in processed foods is far from being ensured. Thus, much research is still required in this field aiming at providing adequate methods for the detection and quantification of food allergens. Multitarget approaches are at the beginning of their development and could represent a high throughput step forward in the detection of hidden allergens in foods.

In summary, in order to protect the health of the allergic individuals, improving their quality of life, members from all relevant sectors (clinicians, researchers, regulatory authorities and food industry) should work in close collaboration to provide solutions for the correct management of food allergies.